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## (54) Group B streptococcal capsular polysaccharides.

(57) Group B Streptococcus type-specific polysaccharides types $I_a$, $I_b$, II and III have been obtained from strains grown in a modified soy bean and yeast medium. These polysaccharides are useful as active or passive streptococcal vaccines.

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TITLE OF THE INVENTION

# Group B Streptococcal Capsular Polysaccharides

## Background of the Invention

Group B Streptococcus (Streptococcus agalactiae) has recently been identified as the leading cause of meningitis among neonates and young infants. A conservative estimation [C.J. Baker, J. Infectious Diseases, 136, No. 1, 137 (1977)] indicates that about 12,000 to 15,000 infants will be affected annually. Half of them will die. Those who survive may develop fatal neurological sequelae that often progress too far to be arrested by antibiotic treatments. For this reason, the immunological, preventive approach as embodied in vaccination of Group B streptococcal type-specific polysaccharides has evolved as one of the most important aspects for future control of the disease. In addition, antibody specific to type $I_a$ Streptococcus agalactiae has recently been found in the glands of bovine immunized with killed or live vaccine, indicating that Group B streptococcal type-specific polysaccharides may be important in vaccination against mastitis in dairy mammals, for example, dairy cattle.

Group B streptococcal antigens are generally classified into five serotypes ($I_a$, $I_b$, $I_c$, II and III) based on capillary precipitin tests with hydrochloric acid-extracted antigens and type-specific hyperimmune rabbit antisera.

Although later studies have shown chemical compositions indicating structures quite different from those reported originally, the same serological classification, i.e., $I_a$, $I_b$, $I_c$, II and III for streptococcal polysaccharides, are still being followed. For example, type-specific polysaccharides isolated via HCl-treatment of whole organisms was reported to contain rhamnose, glucosamine, and galactose, while type-specific polysaccharides prepared by TCA (trichloroacetic acid) extraction contained an additional antigenic determinant, sialic acid. Furthermore, structural difference may also arise from variation of fermentation conditions. Thus Group B Streptococcal polysaccharides isolated by identical methods from organisms grown in the presence of excess glucose has been found to contain no rhamnose as previously reported.

The present invention is related to novel Group B streptococcal polysaccharide types $I_a$, $I_b$, II and III, all of which contain galactose, glucosamine, glucose, and sialic acid. They are structurally distinguishable from those reported earlier as a result of variation in fermentation conditions, methods of isolation and purification.

Accordingly, it is an object of the present invention (1) to provide highly purified antigenic Group B streptococcal polysaccharide types $I_a$, $I_b$,

II and III of novel structures; (2) to provide a vaccine for neonates or infants against Group B Streptococcus infections, for example, meningitis, containing at least one of the novel Group B streptococcal polysaccharides; (3) to provide a method for the prevention of neonatal diseases induced by Group B Streptococcus by vaccination of pregnant women or women of childbearing age; (4) to provide a novel method for the isolation and purification of the Group B streptococcal polysaccharides; (5) to provide a vaccine containing one or more of the type-specific Group B streptococcal polysaccharides for protection against mastitis in dairy mammals; (6) to provide a method for protection against Group B Streptococcus infections by passive vaccination of patients with impaired immune system; and (7) to provide a method for protection against mastitis in dairy mammals by passive vaccination.

Detailed Description of the Invention

Antigenic Group B streptococcal polysaccharide types $I_a$, $I_b$, II and III have been isolated having the specifications shown below in Table I based on dry weight analysis.

TABLE I

SPECIFICATION OF GROUP B STREPTOCOCCUS TYPE-SPECIFIC POLYSACCHARIDES

| | $I_a$ | $I_b$ | II | III |
|---|---|---|---|---|
| $K_d$ | 0.3-0.4 | 0.3-0.4 | 0.3-0.4 | 0.5-0.6 |
| %PRT | 0.5 | 1.0-2.1 | 0.5 | 1.2-5.0 |
| %NA | 0.2 | 0.5 | 0.2 | 0.1-0.5 |
| %P | 0.1-0.5 | 0.1-0.5 | 0.1-0.5 | 0.5 |
| %HXA | 13.7-22.5 | 12.0-18.0 | 10.0-15.8 | 20.0-27.0 |
| %OAC | 0.5 | 0.5-1.0 | 1.0 | 0.5 |
| %HEX | 35.0-40.0 | 50.0-62.5 | 35.0-45.3 | 45.0-55.0 |
| %GLU | 14.1-20.7 | 30.0-41.6 | 15.0-22.1 | 10.0-17.4 |
| %SA | 25.0-30.0 | 7.0-16.6 | 20.6-25.0 | 25.0-35.0 |
| %MP | 0.5-1.0 | 1.4 | 2.0-5.0 | 0.5-1.0 |

$K_d$= partition coefficient in Sepharose 4B gel chromatography

PRT= Protein

NA= Nucleic Acid

P= Phosphorus

HXA= Hexosamines (glucosamine)

OAC= O-acetates

HEX= Hexoses (including galactose and glucose in the ratio of 2:1)

GLU= Glucose

SA= Sialic Acid

MP= Methylpentose

The most purified antigens of this invention have the following analyses. In all cases, the hexosamine is glucosamine and the hexoses are galactose and glucose. $K_D$ values are as determined on Sepharose 4B by gel filtration.

Type $I_a$ (product No. 11059-113)
42.9% hexose, 27.6% hexosamine, 29.3% sialic acid; $K_D$ = 0.22

Type $I_b$ (product No. 11059-193)
42.6% hexose, 18.3% hexosamine, 26.8% sialic acid, $K_D$ = 0.35

Type II (product No. 11059-176)
50.9% hexose, 14.2% hexosamine, 23.9% sialic acid, $K_D$ = 0.44

Type III (product No. 11059-179)
39.9% hexose, 23.3% hexosamine, 28.7% sialic acid, $K_D$ = 0.59

These type-specific antigens can therefore be defined as consisting essentially of polysaccharides of varying composition and molecular weights.

Type $I_a$ is composed of hexose, glucosamine, and sialic acid in the approximate molar ratio 3:1:1, having a molecular weight of about $0.8 \times 10^6$ daltons.

Type $I_b$ contains the same components in the approximate molar ratio 3:1:1, having a molecular weight of about $0.5 \times 10^6$ daltons.

Type II contains the same components in the approximate molar ratio 5:1:1, having a molecular weight of about $0.5 \times 10^6$ daltons.

Type III contains the same components in the approximate molar ratio 3:1:1, having a molecular weight of about $0.15 \times 10^6$ daltons.

The purified type-specific polysaccharides be they type $I_a$, $I_b$, II or III, are composed of galatose, glucosamine, glucose and sialic acid. Very small amounts of protein, nucleic acid, phosphorus, uronic acid and acetate are also present.

The polysaccharides can be isolated from any group B type-specific strain, but preferably from the following strains which are on unrestricted deposit in the American Culture Collections (ATCC).

| Type | Merck No. | ATCC No. |
|---|---|---|
| $I_a$ | MB-4052 | 31,574 |
| $I_b$ | MB-4053 | 31,575 |
| II | MB-4055 | 31,576 |
| III | MB-4082 | 31,577 |
| | MB-4316 (M 732) | 31,475 |

The most preferable strain for type III is M-732.

The preparation of antigenic polysaccharides generally comprises (1) inoculating the bacteria into a modified HySoy nutrient medium containing an excess amount of glucose (about 2.5 to 5% by weight); (2) separating the cell paste from the supernatant after phenolizing the fermentation broth; (3) isolating the crude type-specific polysaccharide either by chemical precipitation or by precipitation after enzyme digestion; and (4) purifying the crude polysaccharides by ammonium or sodium sulfate precipitation (types $I_a$ and $I_b$ only) followed by, if desired, column chromatography with an appropriate gel material as the stationary phase. The commonly used gel materials including Bio-Gel P-300, Sepharose 4B, Sepharose 6B, Whatman's

DEAE-cellulose (DE-52) or the like are found to be satisfactory.

Inoculation of the bacteria is accomplished by culturing a Group B Streptococcus type-specific strain, for example, MB-4055, (type II, ATCC No. 31576) in an inoculum medium such as Fluid Thioglycollate Medium (FTM). The FTM culture obtained is in turn used to inoculate a second inoculum medium which is free of animal protein and contains the necessary nutrients for the growth of the bacteria. For example, the preferred medium contains about 15 to 25 g per liter Hysoy (Humko Sheffield), about 5 to 15 g per liter Amberex 1003 (Amber), about 2.5 to 7.5 g per liter sodium chloride or potassium chloride, about 1 to 5 g per liter potassium or sodium phosphate dibasic ($K_2HPO_4$ or $Na_2HPO_4$), about 0.01 g per liter phenol red and about 10 to 50 g per liter glucose in distilled water at pH 6 to 8, preferably 6.8 to 7.4. After a subsequent 9-liter inoculation, the culture which is assured of its purity according to conventional methods (see Example 1, infra), is inoculated for the large scale production fermentation. The production medium is essentially the same as the inoculum medium except that phenol red is displaced with 8% UCON LB 625 solution (see Table II, for definition) to prevent foam formation.

The more preferable medium is the medium which contains about 20 g per liter HySoy, about 5 g per liter sodium chloride, about 2.5 g per liter potassium phosphate dibasic, about 10 g per liter Amberex 1003 and about 25 g per liter (about 2.5%

by weight) glucose in apyrogenic water at pH 6.9 to 7.4.

More specifically, the most preferable medium for type III polysaccharide contains the same amount of ingredients as described above. However, a solution of HySoy and Amberex 1003 (yeast extract) in about 50 ml of distilled water is dialyzed first with a molecular weight cut-off at about 12,000 to 14,000 before the resulting dialysate is mixed with other components, diluted to about 750 to 1000 ml with apyrogenic water, and adjusted to pH 7.2 before sterilization.

Generally the fermentation is conducted at about 34 to 39°C, preferably at 37°C, until the fermentation is complete, usually from about 3 hours to about 48 hours. Under optimum conditions, the fermentation is over within 24 hours.

After the fermentation is complete, it is stopped by addition of about 0.5 to 2.0% by weight of phenol followed by collection of the cells via centrifugation or filtration, preferably by centrifugation. In most cases, the cell paste is discarded except where enzymatic digestion is used to prepare polysaccharide type $I_a$ or $I_b$ from the cells (see Example 2, infra). The preferable method in-

volves (1) treating the supernatant or filtrate with a strong ionic salt such as calcium chloride, sodium sulfate, sodium chloride, or potassium chloride, preferably calcium chloride; (2) diluting the solution with a lower alkanol such as methanol, ethanol, propanol, or butanol, preferably ethanol (20 to 50% by volume) to precipitate most of the inactive impurities such as nucleic acids; and (3) precipitating from the supernatant obtained above the desired crude type-specific polysaccharide by addition of a water-miscible lower alkanol, preferably ethanol (35 to 75% by volume).

The most preferable conditions for removal of impurities and precipitation of the crude type-specific polysaccharides including types $I_a$, $I_b$, II and III are as follows:

| Removal of Impurities | Precipitation of Crude Polysaccharide |
|---|---|
| 0.1M $CaCl_2$ in 40-44% by volume aqueous ethanol | $CaCl_2$ (ca. 0.07M) in 44-61% by volume aqueous ethanol |

Although each crude type-specific polysaccharide may be precipitated under substantially similar conditions as shown above, further purifications requires different procedures. Crude types $I_a$ and $I_b$ polysaccharides are further purified by (1) suspension in an appropriate amount of an aqueous solution of an inert salt, for example, about 0.5% to 5% by weight of aqueous sodium acetate or ammonium acetate or the like, preferably 1% by weight aqueous sodium acetate; (2) removal of insoluble impurities by centrifugation or filtration; and (3) saturation of

the supernatant with an inert salt such as sodium sulfate, potassium sulfate, or ammonium sulfate, preferably ammonium sulfate, to salt out the semi-purified polysaccharide.

The semi-purified polysaccharide types $I_a$ and $I_b$ are subsequently purified by gel chromatography. Any commonly used gels for chromatography can be used. For example, dextran gels, polyacrylamide gels, agarose gels, macroreticular polystyrene gels, cross-linked polymethylmetaacrylate gels, Bio-gel p-300 (Bio Rad Laboratories, Richmond, California), and Sepharose® 4B or 6B (Pharmacia Fine Chemicals). The preferred gel for isolating types $I_a$ and $I_b$ is Sepharose® 6B or 4B. Generally the semi-purified material is extracted with a minimum amount of about 0.5 to 2.5% aqueous sodium acetate, preferably about 1% by weight aqueous sodium acetate. After centrifugation, the clear supernatant is eluted with an aqueous solution of about 0.5 to 2.5% (preferably 1%) by weight sodium acetate, containing about 0.02% by weight sodium borate, sodium azide or other preservatives. The pH of the eluant is preadjusted to about 7.0. Active fractions determined by the Ouchterlony double immunodifussion technique are combined and the pooled material is concentrated to an appropriate volume at a moderate temperature preferably at 25° C. To obtain the highly purified polysaccharide, the concentrate is first dialyzed against distilled water for about 4 hours. A second dialysis against 0.01 to 0.03% by weight aqueous sodium acetate, preferably 0.02%, is conducted at about 4°C for about 10 to 48 hours or until the

dialysis is substantially complete. The dialyzed concentrate is freeze-dried to afford the purified product.

Alternatively, type $I_a$ or $I_b$ polysaccharide may be purified by the formation of cetavlon (hexadecyltrimethyl ammonium bromide) complex. Under this procedure, the concentrated, combined active fractions are treated with a sufficient amount of cetavlon to form a gel-like precipitate which in turn is dissolved in a minimum volume of 1.0 to 10% by weight, preferably 5% by weight aqueous sodium acetate. About 2 to 4 volumes of water-miscible alcohol, preferably 3 volumes of ethanol are added to precipitate the purified polysaccharide.

The purified polysaccharide, if desired, may be further purified by (1) dissolution in a buffer solution at pH about 8 to 9, preferably a borate buffer at 8.5, and (2) elution through a cellulose-packed column, for example, a Whatman's DEAE-Cellulose (DE-52) column. Active fractions are pooled, concentrated and dialyzed. The resulting retentate is freezed-dried to afford the final product.

As to types II and III, crude polysaccharides are precipitated similarily as types $I_a$ and $I_b$. However, after the initial precipitation, the crude product is suspended in an appropriate amount of an aqueous solution of an inert salt, for example, about 0.5% to 2.5% by weight aqueous sodium acetate or ammonium acetate, preferably 0.1% aqueous sodium acetate. A sufficient amount of trypsin (about 1 mg to 10 mg/100 ml of the suspension) is added and the mixture is incubated at about 34°C to 38°C, preferably at 37°C, for about 0.5 to 2 hours or until the digestion is substantially complete. During the incu-

bation, the pH is maintained at about 8.0 to about 8.5. Semipurified polysaccharides types II and III are subsequently obtained by the calcium chloride-ethanol precipitation as described above for types $I_a$ and $I_b$. For further purification, column chromatography with an agarose gel, for example, Sepharose® 4B, Sepharose® 6B, or Bio-gel P-300 is used. The eluate is subsequently treated with calcium chloride-ethanol to precipitate the polysaccharide which in turn is dissolved in a minimum amount of basic buffer solution of pH at about 8 to 9, preferably a borate buffer at pH 8.5. The resulting solution is eluted through a cellulose-packed column, for example, a column packed with a sufficient amount of wet Whatman's DEAE-cellulose (DE-52). Active fractions are pooled and concentrated by evaporation of the solvents followed by dialysis. The dialyzed concentrate is usually freeze-dried to afford the purified polysaccharide as a white powder.

A vaccine for humans may be prepared by incorporating an effective amount of one or more purified Group B Streptococcus type-specific polysaccharides into a suitable physiologically acceptable medium, for example, saline, water, or phosphate buffered saline.

The dosage of a monovalent vaccine and frequency of administration vary according to the age and physical condition of a patient and is up to the judgment of the clinician.

Usually the dose varies from about 25 μg to about 250 μg of the polysaccharide in about 0.2 ml to 1.0 ml sterile saline or the like. Generally, one or two subcutaneous administrations are sufficient to provide adequate protection against Group B Streptococcus infections.

In some cases, in the judgment of the clinician, it may be advantageous to prepare a polyvalent vaccine containing an effective amount of two or more selected types of Group B streptococcal type-specific polysaccharides, for example, a trivalent vaccine containing types $I_a$, II and III or a tetravalent vaccine containing types $I_a$, $I_b$, II and III. Dosage and administration of a polyvalent vaccine is substantially similar to that of a monovalent vaccine subject to the judgment of the clinician. Generally, the amount of each polysaccharides is from about 25 μg to 250 μg per dose.

Similar dosages are used in administrating a vaccine against mastitis in dairy mammals especially

dairy cattle. However, an adjuvant--the role of which is to assure high immune response--is usually required. The adjuvant may be any compound or composition selected from the group consisting of aluminium hydroxide and aluminium phosphate; for example, ALHYDOGEL®, (Superfos Export Co., Copenhagen, Denmark).

Alternatively, protection against Group B Streptococcus induced infection can be established by passive vaccination, i.e., by giving preformed antibody or homologous γ-globulin from another individual of the same or a different species. The preformed antibody or γ-globulin used for passive vaccination is usually obtained from another individual who was vaccinated actively with the vaccines of the present invention.

The following examples illustrate the present invention.

## EXAMPLE 1

## FERMENTATION OF STREPTOCOCCUS GROUP B BACTERIA (NON-DIALYZED MEDIUM)

Step A: Culture

Streptococcus Group B type $I_a$, $I_b$, II, and III were received by Merck's Rahway Stock Culture Collection (RSCC) from Dr. R. Lancefield, Rockefeller University, New York, New York and were designated as Merck MB-4052, MB-4053, MB-4055 and MB-4082, respectively. The cultures were preserved as lyophilized cultures in the RSCC and have been deposited in the American Type Culture Collection (ATCC) and thereby assigned ATCC numbers 31574,

31575, 31576 and 31577, respectively. Another Type III culture, M-732 (ATCC No. 31,475), which is available on unrestricted deposit with the American Type Culture Collection, Rockville, Maryland, was designated as Merck MB-4316.

Step B: Inoculum Development

A lyophilized "L" tube containing one of the cultures (type $I_a$) obtained from the RSCC was suspended in 1.0 ml of BBL Fluid Thioglycollate Medium (FTM, see Table I, 1, on pg. 17) and 1.0 ml of the resulting suspension was transferred into a test tube containing 9 ml of FTM and incubated at 37°C for 6 hours. After incubation the culture was examined microscopically and streaked on YED (see Table II, 2) plates to check for purity. Small colonies typical of Group B Streptococcus were observed on the YED plate; and numerous streptococcal chains typical of Group B Streptococcus were also observed microscopically.

Five ml of the FTM culture obtained above was used to inoculate 1 liter of inoculum medium (see Table II, 3) in a 2-liter Erlenmeyer flask. The flask was incubated stationarily for about 9 to 12 hours at 37°C and about 4 to 12 hours at 4°C. The pH of the fermentation was adjusted periodically by the addition of 12% aqueous sodium bicarbonate. The 1 liter-fermentation utilized about 80 to 100 ml of the sodium bicarbonate solution.

Before the next inoculation, a sample was (1) applied on a YED plate to check its purity; (2) observed microsocpically; and (3) examined for precipitin reaction with Group B streptococcal antiserum (see

Table II, 6). Small colonies, typical of Group B Streptococcus, were viewed on the YED; the cells were streptococcal formation when examined microscopically; and a single precipitin reaction with Group B antiserum was observed on the Ouchterlony plates.

The culture which was assured of its purity was used to inoculate a 14-liter fermentor (MA 114 - New Brunswick Scientific, Edison, New Jersey) containing 9 liters of inoculation medium (see Table II, 4). The batch was incubated at 37°C with mild agitation (100 rpm) without aeration. Throughout the cultivation, samples were examined for optical density (O.D.) and pH values. The final O.D. before inoculating the production stage was about 2.0 to 5.0. The pH was maintained at about 7.0 during fermentation by the periodical addition of 10% aqueous sodium hydroxide. After the final sodium hydroxide addition, the fermentation was terminated (total time 3 hrs.). A total of 150 ml of aqueous sodium hydroxide (10%) was utilized.

Before inoculating the production fermentor, samples again were taken for purity (YED), Group B specificity (precipitin reaction on Ouchterlony), and microscopic examination. Based upon these examinations, which revealed only streptococcal forms, the production-fermentor was inoculated as described in the next step.

Step C: Production of Fermentation Broth

Approximately 10 liters of the culture obtained from Step B was used to inoculate a 250-liter fermentor (FM 250, New Brunswick Scientific) containing 175 liters of production medium (see Table II, 5). The conditions for growth were 37°C, 100 rpm agitation, and no aeration. Throughout the cultivation

the pH was adjusted to 7.0 with 10% aqueous sodium hydroxide and samples were taken at 2 hr. intervals for optical density (O.D.) and pH measurements. When the pH remained constant at about 7.0 without further additions of sodium hydroxide, the fermentation was stopped.

Prior to harvesting, the culture was plated on YED, examined microscopically, checked by Gram stain (see Table II, 7), and examined for group specificity by Ouchterlony reaction. There were small colonies on the YED plate; the cells were in streptococcal formation when observed under the microscope; the Gram stain was positive; and a single precipitin reaction occurred. These results indicated that the fermentation broth was ready for harvest and inactivation. It was inactivated via addition of phenol (1% by weight).

Following substantially the same procedure as described in Example 1, but substituting for the type $I_a$ culture used therein culture type $I_b$, II, or III, there was obtained the corresponding type $I_b$, type II or type III fermentation broth.

TABLE II

DEFINITION

1. Fluid Thioglycollate Medium (FTM)

| | |
|---|---|
| Fluid thioglycollate powder | 29.5 g/liter |

2. YED Plates

| | |
|---|---|
| Amberex 1003 (Amber) | 10 gm/liter |
| Dextrose | 10 gm/liter |
| Agar (Difco) | 20 gm/liter |

3. Inoculum Medium (2-Liter Flask)

| | |
|---|---|
| HySoy (Humko Sheffield) | 20 gm |
| Amberex 1003 (Amber) | 10 gm |
| NaCl | 5 gm |
| $K_2HPO_4$ | 2.5 gm |
| Phenol red | 10 mg. |

The above components are dissolved in distilled water and the volume of the solution is adjusted to 900 ml.

The pH is adjusted to 7.2 and the medium is autoclaved for 25 minutes.

Glucose (25 g) in 100 ml of distilled water is autoclaved separately for 20 minutes and added aseptically to the medium.

4. Inoculation Medium (14-Liter Fermentor)

| | |
|---|---|
| HySoy (Humko Sheffield) | 180 gm |
| Amberex 1003 (Amber) | 90 gm |
| NaCl | 45 gm |
| $K_2HPO_4$ | 22.5 gm |
| *UCON LB 625 8% solution | 40 ml |

The above components are dissolved in distilled water and the volume of the solution is adjusted to 8 liters.

The pH is adjusted to 7.2 and the medium is autoclaved for 90 minutes. Glucose (225 g) in 1 liter of distilled $H_2O$ is autoclaved separately for 30 minutes and added aseptically to the medium.

*The UCON LB 625 8% solution is pre-sterilized for 1 hour.

5. Production Medium (250-Liter Fermentor)

| | |
|---|---|
| HySoy (Humko Sheffield) | 3500 gm |
| Amberex 1003 (Amber) | 1750 gm |
| NaCl | 875 gm |
| $K_2HPO_4$ | 437.5 gm |
| UCON LB 625 8% solution | 400 ml |

The above components are dissolved in distilled water and the volume is brought up to 165 liters.

The pH is adjusted to 7.2 and the medium is autoclaved for 30 minutes.

Glucose (4375 g) in 10 liters of distilled water is autoclaved separately for 30 minutes and added aseptically to the medium.

6. Ouchterlony Test for Group B

a. A 5 µl sample of Group B streptococcal antiserum is placed in the center well of an Ouchterlony plate (Hyland).

b. A 5µl sample of streptococcal broth is placed in the outside well.

c. A precipitin reaction occurs if streptococcal Group B cells are present.

7. Gram Strain

A rapid method for detecting shape and cell arrangement. The Gram strain reaction will frequently enable the investigator to narrow down the general identification to a small group.

EXAMPLE 2

FERMENTATION OF TYPE-SPECIFIC GROUP B STREPTOCOCCUS (DIALYZED MEDIUM)

Following substantially similar procedures as described in Example 1, steps A to C, but substituting for the media used therein the corresponding media described below in Table III, there was obtained type $I_a$, type $I_b$, type II or type III fermentation broth ready for harvesting and isolation.

TABLE III

DIALYZED MEDIA FOR DIFFERENT STAGES OF FERMENTATION

1. Inoculum Medium (2-Liter Flask)

| | |
|---|---|
| HySoy (Humko Sheffield) | 20 gm |
| Amberex 1003 (Amber) | 10 gm |
| NaCl | 5 gm |
| $K_2HPO_4$ | 2.5 gm |
| Phenol red | 10 mg |

HySoy and Amberex 1003 are dissolved in 50 ml of distilled water and dialyzed with 12,000 to 14,000 molecular weight cut-off. The resulting dialysate together with NaCl, $K_2HPO_4$, and phenol red are diluted with distilled water to a volume of 900 ml.

The pH is adjusted to 7.2 and the medium is autoclaved for 25 minutes.

Glucose (25 g) in 100 ml of distilled water is autoclaved separately for 20 minutes and added aseptically to the medium.

2. Inoculation Medium (14-Liter Fermentor)

| | |
|---|---|
| HySoy (Humko Sheffield) | 180 gm |
| Amberex 1003 (Amber) | 90 gm |
| NaCl | 45 gm |
| $K_2HPO_4$ | 22.5 gm |
| UCON LB 625 8% solution | 40 ml |

HySoy and Amberex 1003 were dissolved in 450 ml of distilled water and dialyzed with 12,000 to 14,000 molecular weight cut-off. The resulting dialysate together with NaCl, $K_2HPO_4$, and UCON LB 625 are diluted with distilled water to a volume of 8 liters.

The pH is adjusted to 7.2 and the medium is autoclaved for 90 minutes. Glucose (225 g) in 1 liter of distilled $H_2O$ is autoclaved separately for 30 minutes and added aseptically to the medium.

3. Production Medium (250-Liter Fermentor)

| | |
|---|---|
| HySoy (Humko Sheffield) | 3500 gm |
| Amberex 1003 (Amber) | 1750 gm |
| NaCl | 875 gm |
| $K_2HPO_4$ | 437.5 gm |
| * UCON LB 625 8% solution | 400 ml |

HySoy and Amberex 1003 are dissolved in 9 liters of distilled water and dialyzed with 12,000 to 14,000 molecular weight cut-off. The resulting dialysate together with NaCl, $K_2HPO_4$ and UCON LB 625 are diluted with distilled water to a volume of 165 liters.

The pH is adjusted to 7.2 and the medium is autoclaved for 30 minutes.

Glucose (4375 g) in 10 liters of distilled water is autoclaved separately for 30 minutes and added aseptically to the medium

*The UCON LB 625 8% solution is pre-sterilized for 1 hour.

EXAMPLE 3

PREPARATION OF GROUP B STREPTOCOCCUS POLYSACCHARIDE TYPE $I_a$ VIA ENZYMATIC-DIGESTION OF THE CELL PASTE

Step 1: Enzymatic digestion of Cell Paste

The wet cell paste (105 g wet weight) obtained from the phenolized fermentation broth (Example 1) by centrifugation was suspended in 1.6 liters of 0.1 M sodium acetate with a final pH adjustment to 6.5. The resulting suspension was subsequently charged with 0.02% by weight of sodium azide as a preservative. The system underwent lysis with 4,000 azocasein units of Endopeptidase (Calgon Formulation No. 6859 113 & 005B) followed by incubation at 37°C for 36 hr. The resulting digest (11.8 liters) was stored at 2°C. Aliquots were taken (1) for serological studies to confirm the presence of type-specific polysaccharide type $I_a$ by the Ouchterlony double immunodiffusion technique against homologus antisera (Merck, Sharp & Dohme Research Laboratories) and (2) for ethanol fractionation to determine the ethanol ranges which would precipitate the type-specific polysaccharide type $I_a$ with the aid of the serological study described in (1). It was found that 44% (vol/vol) ethanol would effectively precipitate the type $I_a$ polysaccharide.

Step 2: Preparation of Crude Type $I_a$ Polysaccharide

The digest from step 1 (1.8 liters) was treated with an aqueous solution of calcium chloride (1.1 g/ml or 0.1 M). Sufficient amount of ethanol was added at room temperature (about 20-25°C) until the bulk solution contained about 44% ethanol by volume. The resulting precipitates which consisted of impurities such as

calcium nucleinates and cell debris were removed by centrifugation at 5,000 rpm for 10 minutes (Sorvall model RC-2B).

The supernatant was then diluted with ethanol to a concentration of 60% (vol/vol) in ethanol in order to precipitate the crude type $I_a$ polysaccharide. After sufficient time was allowed for the resulting flocculent precipitate to settle, most of the supernatant was siphoned off, and the crude product was collected by centrifugation at 5,000 rpm for 5 minutes. The crude type $I_a$ polysaccharide was suspended with 200 ml of ethanol in a Waring blender. It was subsequently filtered, washed with 1 x 200 ml of ethanol and 1 x 200 ml of acetone. After drying under vacuum in a desiccator over anhydrous calcium chloride overnight, there was obtained 2.42 g of crude type $I_a$ polysaccharide.

Step 3: Purification of Crude Type $I_a$ Polysaccharide by Molecular Sieving Chromatography

The crude type $I_a$ polysaccharide (1 g) in about 10 ml of 0.5% sodium acetate (pH 6.5) containing 0.02% sodium azide (solution A) was chromatographed through a column of Bio-Gel P-300 (Bio Rad Laboratories, Richwood, California) with solution A as eluant at a flow-rate of 0.4 ml/min. The elution was monitored serologically by the Ouchterlony technique. To precipitate the purified product, fractions containing the product were combined and diluted with 3 volumes of isopropyl alcohol. After standing at ambient temperature for about one hour, the supernatant was decanted, and the precipitate was triturated with 200 ml of

ethanol in a Waring blender, followed by filtration and subsequent washes with ethanol (100 ml) and acetone (100 ml). The wet product was dried in vacuo over anhydrous calcium chloride to give 0.74 g of group B Streptococcus polysaccharide type $I_a$.

EXAMPLE 4

PREPARATION OF GROUP B STREPTOCOCCUS POLYSACCHARIDE TYPE $I_a$ FROM THE CELL-FREE BROTH VIA CHEMICAL ISOLATION

Step 1: Preparation of Cell-Free Fermentation Broth

The phenolized fermentation broth (containing 1% by weight of phenol) from Example 1 was cleared of cells by centrifugation in a Sharples Ultracentrifuge Model T-1-P.

The supernatant, 20 liters, was charged with calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) to 0.1 M in $CaCl_2$ and with denatured ethanol to 44% by volume in ethanol. The alcohol was added in 100 ml-lots per minute with adequate stirring at room temperature. The resulting suspension was allowed to stand for 4 hours and subsequently centrifuged in the Sharples Ultracentrifuge. The feeding rate of the suspension was adjusted to about 150 ml per minute to assure clarity of the effluent which was substantially free of nucleic acid and other inactive materials.

Step 2: Precipitation of Crude Type-Specific Polysaccharide

The effluent from Step 1 was charged with additional ethanol until it contained 60% by volume ethanol. A flocculent precipitate formed after standing overnight. The crude material was collected by siphoning off most of the clear supernatant followed

by centrifugation at 6,000 rpm for 10 minutes at 20°C in a Sorvall centrifuge, Model RC-5.

The resulting pellet of crude type-specific polysaccharide was suspended in 300 ml of 1% by weight aqueous sodium acetate. Usually, it required about 4 hours with mild stirring to achieve complete suspension. Insolubles were removed by centrifugation at 10,000 rpm for 15 minutes at 4°C in a Sorvall centrifuge, Model RC-5. The supernatant (325 ml) containing the desired polysaccharide type $I_a$ was treated with 200 g of anhydrous sodium sulfate (86% saturation) with adequate agitation. Since the salted-out material tended to rise to the surface, the entire system was degassed under mild vacuum in order to facilitate the precipitation. After standing overnight at 4°C, the precipitated crude polysaccharide type $I_a$ was collected by centrifugation in the usual manner as described above.

Step 3: Isolation of Group B Streptococcus polysaccharide Type $I_a$ by Sepharose 6B Chromatography

The salted-out material from step 2 was extracted with 30 ml of 1% aqueous sodium acetate to give a viscous solution. The remaining turbidity was removed by centrifugation at 15,000 rpm for 20 minutes at 4°C. The almost clear supernatant (ca. 30 ml) was applied to a Sepharose 6B column with a bed volume of 5.6 x 90 cm preequilibrated with the eluant (1% sodium acetate containing 0.02% sodium azide as a preservative, with a pH of 7.3). Fractions (12.5 ml) were collected with maximal flow rate of ca. 1 ml per minute.

The elution was monitored by (1) refractive index changes (Waters Associates), and (2) by the Ouchterlony double immunodiffusion technique for serological activity. It was found that peak serological activity coincided with a refractive index peak. The serologically active fractions were pooled and was reduced in volume to ca. 30 ml in a hollow fiber device, No. 80, (100 ml beaker, Bio Rad Laboratories, Richmond, Cal.). The ionic strength of the pooled fractions was reduced to less than 0.25% by flushing with apyrogenic water. By backflushing the device with about 70 ml of water, the combined active fractions were efficiently recovered with a total volume of about 100 ml and were used directly in the next step.

Step 4: Further Purification of Group B Streptococcus polysaccharide type $I_a$ via Cetavlon Complex Formation

The pooled active fractions (100 ml) from Step 3 was charged with 300 mg of cetavlon. A glassy gel-like precipitate was formed and was collected at once by centrifugation at 10,000 rpm for 10 min. at 4°C (Sorvall, Model RC-5). To test for the completeness of the complex formation and to assure that the initially existing ionic strength of foreign ions was not too high, the supernatant was dialyzed overnight against a 1% by weight aqueous solution (500 ml) of cetavlon. The completeness of the complex formation was indicated by the lack of further complex formation, i.e., the supernatant stayed clear after the test.

The complex was suspended in 20 ml of 5% sodium acetate to give a clear, slightly viscous, colorless solution. The desired polysaccharide type $I_a$ was

precipitated with 3 volumes of ethanol. A heavy, flocculent precipitate resulted and the supernatant decanted. The precipitate was washed with 250 ml of ethanol to remove any sodium acetate remaining in the precipitate. It was further washed with acetone, and air-dried.

The resulting powder was transferred into a 100 ml round-bottom flask and dissolved in 20 ml of 0.02% sodium acetate. The clear solution was dialyzed against 1 liter of water and the dialyzed concentrate was freeze-dried to afford 205 mg of purified Group B Streptococcus polysaccharide, type $I_a$.

Following substantially the same procedure of Example 4 but substituting for the type $I_a$ specific antiserum used therein for the Ouchterlony double immunodiffussion technique for serological activity, the type $I_b$ specific antiserum, there was obtained 5-10 mg/liter of broth of purified Group B Streptococcus type $I_b$ polysaccharide.

## EXAMPLE 5

PREPARATION OF PURIFIED GROUP B STREPTOCOCCUS POLYSACCHARIDE, TYPE $I_b$

Step 1: Preparation of Cell-Free Fermentation Broth

At the end of the fermentation cycle, the broth was phenolized to contain 1% by volume of phenol. Cells were subsequently removed by centrifugation in a Sharples supercentrifuge, Model T-1-P, and the supernatant was stored at 4°C overnight.

Step 2: Precipitation of the crude Group B Streptococcus Polysaccharide, Type $I_b$

The cold supernatant (10 l) from step 1 was charged with calcium chloride dihydrate until its concentration reached 0.1 M. The temperature of the supernatant rose to about 15°C due to the exothermic solubilization of the salt. Without cooling, a sufficient amount of ethanol was added with stirring until the resulting mixture contained 60% by volume of ethanol and the precipitation of impurities began. After standing at room temperature for 24 hours, the supernatant was decanted, and the precipitate was collected at 20°C by centrifugation for 10 min. at 5,000 rpm. The wet precipitate (280 ml) was suspended in 5 volumes (1400 ml) of 0.5% by weight aqueous sodium acetate followed by treatment with calcium chloride and ethanol to give a final mixture containing 0.1 mole/liter of calcium chloride and 44% by volume ethanol.

The ethanol addition was carried out at a rate of 10 ml/min. with adequate stirring. After standing for 2 hours at ambient temperature, the precipitate (calcium nucleinates) was discarded by centrifugation, and the ethanol content of the clear supernatant was adjusted to 60% by volume with additional amount of ethanol. The resulting suspension was allowed to stand overnight to precipitate most of the desired polysaccharide, type $I_b$. Upon centrifugation, a wet pellet of crude polysaccharide was collected.

Step 3: Precipitation of semi-purified Polysaccharide with Ammonium Sulfate

The pellets were suspended in 300 ml of 1% aqueous sodium acetate. Insolubles were removed by centrifugation at 10,000 rpm for 15 minutes at 4°C in a Sorvall centrifuge, model RC-5.

The volume of the supernatant was noted and the extract partially saturated (85% saturation) with anhydrous ammonium sulfate to salt out the polysaccharide. The suspension was degased in a desicator under vacuum followed by standing at 4°C overnight. The precipitated polysaccharide was collected by centrifugation as described above.

Step 4: Preparation of Purified Polysaccharide type $I_b$ by Chromatography

The precipitates from Step 3 were extracted with 20 ml of 1% aqueous sodium acetate with insolubles removed by centrifugation at 15,000 rpm for 10 minutes and at 4°C. The clear supernatant (10 ml) was applied to a column of Sepharose 6B (2.6 x 90 cm) and developed with 1% aqueous sodium acetate containing 0.02% sodium azide as preservative. Fractions of 12 ml each were collected (flow rate ca. 3 fractions/min.). The elution was monitored for type $I_b$ polysaccharide by the Ouchterlony double immunodiffusion technique. The serologically active fractions were pooled and concentrated to ca. 20 ml by evaporation. The concentrate was dialyzed for 4 hours against 1 liter of distilled water followed by 18 hours against 2 liters of 0.02% by weight aqueous sodium acetate 4°C. The dialyzed concentrate was freeze-dried to afford 235 mg. of purified group B Streptococcus polysaccharide, type $I_b$.

EXAMPLE 6

PREPARATION OF GROUP B STREPTOCOCCUS POLYSACCHARIDE TYPE $I_a$

Employing substantially similar procedures as described in Example 5, Steps 1-4, but using type $I_a$ broth and substituting for the antiserum used therein for monitoring polysaccharide type $I_b$, the appropriate amount of polysaccharide type $I_a$-specific antiserum for detecting the type $I_a$ serologically active fractions, there was prepared 25 mg of purified Group B Streptococcus polysaccharide, type $I_a$.

EXAMPLE 7

PREPARATION OF GROUP B STREPTOCOCCUS TYPE III POLYSACCHARIDE

Step 1: Isolation of Crude Group B Streptococcus Type III Polysaccharide

Employing essentially the same procedures of Example 4, Steps 1 and 2, there was obtained the crude type III polysaccharide precipitate from the phenolized fermentation broth of Example 1. It was suspended in 200 ml of 1% by weight aqueous sodium acetate (pH 8.5) to give a slightly cloudy solution. Five milligrams of highly purified trypsin (Wortington Co., Freehold, N.J.) were added followed by incubation at 37°C for 1 hr. pH value of the solution was maintained at 8.0 to 8.5 during the incubation period. The resulting digest was cooled to about 0-10°C. Precalculated amount of solid calcium chloride and ethanol were added so as to make the final concentration of the solution 0.1 M in calcium chloride and 61% (v/v)

in ethanol. A suspension resulted. It was allowed to stand overnight for complete precipitation. After the clear supernatant was decanted, the precipitates were collected by centrifugation (5 minutes at 5,000 rpm, 20°C).

Step 2: Purification of Group B Streptococcus Polysaccharide Type III

The crude precipitates obtained in step 1 was extracted with 10 ml of 1% aqueous sodium acetate. After centrifugation, the clear supernatant was applied to a 2.6 x 90 cm Sepharose 6B column (Pharmacia Co., Piscataway, N.J.) and was eluted with 1% by weight aqueous sodium acetate containing 0.02% by weight of sodium azide, a preservative agent. Twelve milimeter fractions were collected at a flow rate of 3 fractions per hour. The elution was followed by Ouchterlony double immunodiffusion techinque against type III specific rabbit antiserum and serologically active fractions were identified and pooled. It was diluted with four volumes of ethanol to precipitate semi-purified type III polysaccharide which in turn was centrifuged upon completion of the precipitation. The pellet was successively washed with ethanol and acetone. It was dried under vacuum in the presence of anhydrous calcium chloride to afford 65 mg of semi-purified product.

Step 3: Final Purification of Group B Streptococcus Polysaccharide Type III by DEAE-Cellulose Column Chromatography

The semi-purified polysaccharide from step 2 was dissolved in 100 ml of 0.05 M borate buffer (pH 8.5) and applied to a 1.0 cm x 30 cm column packed with 20 g of wet Whatman's DEAE-cellulose (DE-52) pre-equilibrated with 0.05 M sodium borate at pH 8.5.

Under these conditions the type III polysaccharide will associate with the cellulose, while remaining impurities will wash through. For this reason, the DEAE-cellulose column was washed with 200 ml of 0.05 M sodium borate (pH 8.5) to remove most of the impurities. Subsequently, the type III polysaccharide associated with the cellulose was eluted with 0.2 M aqueous sodium chloride in 0.05 M sodium borate buffer at pH 8.5. Fractions of 5 ml were collected and the emergence of the type III polysaccharide was monitored by the Ouchterlony double immunodiffusion technique against type III-specific antiserum. Active fractions were pooled and were concentrated by evaporation to about 20 ml and the concentrate was dialyzed for 24 hours against 0.02% by weight sodium acetate at 4°C. The dialyzed concentrate was freeze-dried to give 35 mg of purified Group B Streptocuccus polysaccharide type III.

Following substantially the same procedure as described in Example 7, but substituting for the type III-specific antiserum used therein for monitoring polysaccharide type III, the type II-specific antiserum for detecting the serologically active fractions, there was prepared 230 mg of purified Group B Streptococcus polysaccharide, type II.

## EXAMPLE 8

PREPARATION OF GROUP B STREPTOCOCCUS TYPE III POLYSACCHARIDE FROM CELL-FREE BROTH OBTAINED FROM FERMENTATION IN DIALYZED MEDIUM

Following substantially similar procedures as described in Example 7, Steps 1 to 3, but substituting

for the phenolized fermentation broth used therein, the fermentation broth obtained from Example 2, there was prepared 300 mg of purified Group B Streptococcus polysaccharide type III.

EXAMPLE 9

VACCINE - DOSAGE FORM

One tenth gram of the purified polysaccharide type III was suspended in 500 ml of pyrogen-free distilled water. The suspension was sterile filtered and packed in vials, 5 ml per vial for 10 doses or 100 μg per dose. The vaccine so obtained may be administered subcutaneously or intramuscularly to expecting mothers or women of child bearing age.

EXAMPLE 10

VACCINE - DOSAGE FORM

Two tenth grams each of the purified polysaccharide types II and III were added with sufficient stirring and under aseptic conditions to 1.5 liters of pyrogen-free saline (0.85% by weight). After the addition, the mixture was stirred at room temperature for an additional 2 to 5 minutes, and sterile filtered. The clear filtrate was distributed into 10 ml vials which were either sealed or tightly stopped under aseptic conditions. Each vial contained 5 ml of the filtered vaccine corresponding to 10 doses (about 60 to 65 μg/dose) for injection.

EXAMPLE 11

POLYVALENT VACCINE

A polyvalent vaccine was prepared by adding under aseptic conditions at room temperature the following amounts of capsular polysaccharide to 4 liters of pyrogen-free saline (0.85%). The polysac-

charides were added individually at 15 second intervals while stirring continuously in a blender. After the addition was complete, the stirring was continued for another 2 to 3 minutes.

| Type | Amount (gram) |
|---|---|
| $I_a$ | 1.6005 |
| $I_b$ | 0.8210 |
| II | 0.6005 |
| III | 1.2400 |
| | 4.2620 |

The mixture was subsequently sterile filtered and packaged in vials, 5 ml (10 doses) per vial, i.e., about 75 µg (type II) to 200 µg (type $I_a$) per dose.

In Examples 12-15, the following reagents are used:

Acetone, Baker Analyzed

Ammonium Sulfate, Fisher Scientific Co. or Schwarz/Mann

Boric Acid, Merck & Co.

Calcium Chloride, Dihydrate, Baker Analyzed

Cetavlon (Hexadecyltrimethyl-ammonium Bromide), Eastman Kodak - a cationic detergent

DEAE-Cellulose (DE-52), Whatman Ltd.

Ethanol, Denatured (toluene), Merck & Co.

Ethanol, Denatured, 2BA, Type T, Merck & Co.

Ethanol, 200 Proof, U.S. Industrial Chemicals Co.

Glacial Acetic Acid, Merck & Co.

Sepharose 6B, Pharmacia

Sodium Acetate, Anhydrous, Mallinckrodt

Sodium Chloride, Baker Analyzed

Sodium Hydroxide, 50% Solution, Baker Analyzed

Trypsin, Worthington, 238 units/mg.

Reagents are prepared with triply distilled water. Likewise all containers are rinsed in this water.

## EXAMPLE 12

## RELEASE PROTOCOL FOR GROUP B STREPTOCOCCUS TYPE Ia POLYSACCHARIDE, PRODUCT 11059-113

Step 1 - Isolation of First Crude Product

Sixty (60) liters of whole, phenolized broth were charged with 885 gm of calcium chloride, dihydrate to 0.1 M. Ethanol (denatured) was then added to the 44% vol/vol level. The suspension was allowed to stand for maximal settling of the cells

and other insolubles (about 48 hours). The clear supernatant was then transferred into another fractionation vat, with a recovery of 73 liters. Of this volume, 41 liters represent the aqueous phase. On this basis 29 liters of ethanol (denatured) were added to raise the ethanol level to 60%. Again, the suspension was allowed to stand for maximal settling of the precipitate which contains the desired polysaccharide. The clear supernatant was then pumped off and the precipitate collected by centrifugation (Beckman, Model J-21C, in 500 ml cups, for 15 minutes at 6,000 RPM and at 20°C).

Step 2 - Extraction of the Polysaccharide

The rubbery pellets were suspended with the aid of a blender in 1800 ml of 1% sodium acetate with pH adjustment to 8.5 (using 2 N sodium hydroxide). The suspension was then cleared by centrifugation (as in Step 1, except for 30 minutes at 8,000 RPM). There was a firm pellet, overlaid, however, by a creamy paste, which made it necessary to aspirate off the clear supernatant as decantating was not possible. The total volume was 1,240 ml extract.

Step 3 - Removal of Proteinaceous Materials with Trypsin

The extract from Step 2 was charged with 30 mg trypsin and incubated at 37°C for 90 minutes. The pH was monitored for the range 8.2 - 8.6 during the incubation period. The digest was then immediately placed in an ice-bath.

Step 4 - Ammonium Sulfate Precipitation of the Polysaccharide

While in an ice-bath the digest was charged with ammonium sulfate (750 gm) to 86% saturation. The resulting suspension was allowed to stand at 4°C overnight to precipitate the polysaccharide. The clear supernatant was removed by aspiration and the salted out material was collected by centrifugation as described in Step 1.

Step 5 - Chromatography on DEAE-Cellulose

The ammonium sulfate precipitate from Step 4 was dissolved in 200 ml 1% sodium acetate and exhaustively dialyzed for 48 hours (2 x 12 hours against 10 liters distilled water, followed by 24 hours against 10 liters of 0.05 M borate buffer, pH 8.5). A light turbidity in the retentate was cleared by centrifugation (Sorvall, Model RC-5, in 50 ml cups, at 15,000 RPM for 20 minutes at 4°C). The clear supernatant fluid was then applied to a column of DEAE-Cellulose, equilibrated with sodium borate (0.05 M, pH 8.5), (dimensions of 5.0 x 30 cm). The column was then washed with 700 ml borate buffer. Sodium chloride (0.3 M) in borate buffer, 800 ml, was then applied to a column to mobilize the adhering polysaccharide. One hundred milliliter (100 ml) fractions were collected and separately assayed serologically for their polysaccharide content. Active fractions (2-8) were pooled and the pool treated with 1.5 volumes of ethanol (denatured) to precipitate the polysaccharide which was recovered after removal of the clear supernatant and by centrifugation (as per Step 1). Pellet volume was estimated as 10 $cm^3$.

Step 6 - Recovery of the Polysaccharide as a Cetavlon Complex

The precipitate from Step 5 was resuspended in 300 ml triply distilled water to give a clear solution. One hundred milliliters (100 ml) of a 3% solution of Cetavlon in water was then added with stirring. The polysaccharide aggregated into a single lump allowing its easy removal.

Step 7 - Recovery of Product Intermediate 11059-101

The complex from Step 6 was solubilized in 200 ml 15% sodium acetate, at pH 8.0 at 4°C. It took several hours to achieve complete solubilization. The solution was finally charged with two volumes of ethanol (denatured) to precipitate the polysaccharide as its sodium salt. The precipitate was collected, after removal of the clear supernatant, by centrifugation, as per Step 1. The pellet was triturated with about 100 ml ethanol (denatured) in a blender then collected on a small sintered glass funnel (15 ml, M), washed thereon with 50 ml ethanol and 50 ml acetone, followed by drying in a vacuum desiccator. The product intermediate was called 11059-101, (yield 805 mg).

Step 8 - Molecular Sieving on Sepharose 6B and Final Product Recovery

Two hundred milligrams (200 mg) of 11059-101 were dissolved in 12 ml column buffer (1% sodium acetate) and the solution applied to a column of Sepharose 6B (2.6 x 90 cm) equilibrated with 1% sodium acetate. Fractions of 12.5 ml were collected and active fractions were identified by the Ouchterlony double immunodiffusion method, using a

type specific antiserum. Active fractions (14-29) were pooled. The operation was then repeated with a second 200 mg portion of 11059-101. The 2 pools were combined and concentrated in a Bio Rad No. 80 hollow fiber device to 100 ml. The concentrate was treated with 2 volumes of ethanol (200 proof) to precipitate the polysaccharide. The precipitate was allowed to settle out and recovered, after removal of the supernatant and by centrifugation as per Step 1. The precipitate was then triturated with 50 ml ethanol (200 proof) in a blender and was collected on a small sintered glass funnel (15 ml, M), washed thereon with 50 ml ethanol (200 proof) and 50 ml acetone, followed by drying in a vacuum desiccator to constant weight. Final product: 11059-113 (yield 300 mg).

EXAMPLE 13

RELEASE PROTOCOL FOR GROUP B STREPTOCOCCUS TYPE Ib POLYSACCHARIDE, PRODUCT 11059-186

Step 1 - Preparation of Cell-Free Fermentation Broth

Type Ib broth, phenolized to 1% at termination of the fermentation cycle, was cleared of cells by centrifugation in a Sharples ultracentrifuge, Model T-1P.

Step 2 - Ultrafiltration/Diafiltration of Cell-Free Broth

Sixty liters (60 liters) of the cell-free supernatant were pumped through an ultrafiltration hollow fiber device (Amicon, Model DC-2; molecular weight cut-off of fibers of 50,000 daltons) to a final volume of 11.29 liters.

Step 3 - Isolation of First Crude Product

The concentrate was charged with calcium chloride to 0.1 M and subsequently with ethanol (denatured 2 BA, T type) to 30% by volume. The resulting suspension was centrifuged in a Sharples centrifuge to obtain a clear supernatant (15.13 liters). The ethanol level was then raised to 61% by the addition of 12.06 liters of ethanol (denatured). The resulting precipitate was obtained by allowing it to settle out, removing of most of the clear supernatant, and finally by centrifugation (Beckman, Model J-21C, 50 ml-cups, 15 minutes, 20°C, 6,000 RPM).

Step 4 - Isolation of Second Crude Product

The first crude was suspended in 1,800 ml of 3% sodium acetate and full solubilization assured by stirring the suspension vigorously overnight at 4°C. A serological probe performed on a small aliquot of the suspension indicated that the desired polysaccharide is fractionable with ethanol in the 40-60% range. The total volume of the suspension was 1,980 ml. The suspension was accordingly charged with 1,220 ml ethanol (denatured) to the 40% level and the alcoholic suspension cleared by centrifugation in the Beckman as described in Step 3. A considerable amount of insoluble materials were removed at 40% ethanol. The clear supernatant was then charged with additional (1,650 ml) ethanol (denatured) to 60%, and the resulting suspension was allowed to stand for 48 hours to let the insolubles settle out. The insolubles (second crude) were then recovered by removal of most of the supernatant and by centrifugation as already described.

Step 5 - Isolation of the Polysaccharide as a Cetavlon Complex

The wet pellets, ca. 40 cc in volume, were dissolved in 250 ml water to give a clear, but pigmented solution. The solution was then dialyzed for 24 hours to remove traces of sodium acetate. The retentate was then charged with 100 ml of a 5% solution of Cetavlon in water. The resulting suspension was let stand at 4°C for 18-20 hours to allow the complexing to go to completion. A small aliquot of the suspension was cleared by centrifugation and the supernatant checked for completion of complexing by adding more Cetavlon to the clear supernatant. There was no further precipitate. The Cetavlon-polysaccharide complex was then collected by centrifugation in a Sorval centrifuge, Model RC-5, in 50 ml cups, for 10 minutes at 15,000 RPM and at 4°C. The pellet was not entirely firm, but with a consistency of molasses allowed of the quick decantation of the clear supernatant.

Step 6 - Recovery of the Third Crude Product

The Cetavlon complex was extracted with 200 ml of a 15% solution of sodium acetate at pH 8.0 and the cleared extract (by centrifugation) charged with 2 volumes of ethanol (denatured). A gum formed which settled out overnight, allowing the total decantation of the alcoholic supernatant.

Step 7 - De-Ionization of the Third Crude Product

The third crude was taken up in 200 ml 0.05 M borate buffer, pH 8.5 and ultrafiltered in an Amicon stirred cell (200 ml capacity, equipped with a 30,000 dalton cut-off membrane). To remove traces of

sodium acetate the volume was reduced to 50 ml then increased to 200 ml with borate buffer, followed by reduction to 50 ml.

Step 8 - DEAE-Cellulose Fractionation of the Third Crude Product

The retentate from Step 7 was applied to a column of DEAE-cellulos (5.0 x 30 cm), equilibrated with 0.5 M borate buffer, pH 8.5. The column was washed with 700 ml borate buffer. The wash did not give a precipitate with 1.5 volumes ethanol, nor did it contain any trace of serologically active material in a test with type specific antiserum (Ib) by the Ouchterlony double immunodiffusion method. The column was then washed with 0.25 M sodium chloride in borate buffer and 100 ml fractions collected. Fractions containing serologically active material (No. 4-10) were pooled, and the polysaccharide was precipitated with 1.5 volumes of ethanol (denatured). After the precipitate had settled out, most of the supernatant was removed by aspiration and the precipitate was finally collected by centrifugation (Sorvall, as described in Step 5). This material was designated the Fourth Crude.

Step 9 - Trypsin Digestion of Fourth Crude Product to Obtain the Fifth Crude Product

The precipitate from Step 8 was dissolved in 150 of a soluton of 3% sodium acetate and the pH adjusted down to 8.5. A blender was used to speed up the dispersion of the material. Trypsin was added (20 mg; 238 units/mg), and the system incubated for 90 minutes at 37°C. The pH was monitored from time to time, but found unchanged throughout the

incubation cycle. The digest was then charged with 1.5 volumes of ethanol (denatured) and the precipitate collected by centrifugation in a 500 ml cup (Beckman centrifuge). The pellet was triturated in a small blender with absolute ethanol, collected on a small sintered glass funnel (15 ml, M), washed thereon with 50 ml absolute ethanol and 50 ml of acetone and finally dried in a vacuum desiccator overnight. A white powder was obtained, product intermediate (Fifth Crude) 11059-185, with a yield of 741 mg.

Step 10 - Purificatin with Ammonium Sulfate

Product intermediate 11059-185, as shown by Sepharose 6B profiling, still carries a considerable amount of contaminant, masking in part the type polysaccharide. It was therefore decided to precipitate the desired polysaccharide with ammonium sulfate, at 86% saturation. The lot of the product intermediate was dissolved in 100 ml triply distilled water, and the solution was charged with enzyme grade ammonium sulfate (61 gm). The resulting suspension was let stand in the cold for 2 hours, and the precipitate was then recovered by centrifugation (Sorvall, 15,000 RPM, 50 ml cups, for 15 minutes at 2°C).

Step 11 - Molecular Sieving of Ammonium Sulfate Precipitate

The pellets from the previous step were dissolved in 12 ml eluant (1% sodium acetate, pH 7.5). The clear solution, with a pale-yellow pigmentation, was then applied to a column of Sepharose 6B, equilibrated with 1% sodium acetate, pH

7.5, (dimensions of 2.6 x 90 cm). Fractions of 12.5 ml were collected, and their polysaccharide content monitored serologically by Ouchterlony double immunodiffusion technique. Active fractions were pooled.

Step 12 - Recovery of Final Product, 11059-186

The pool (213 ml) from Step 11 was diafiltered and concentrated to 20 ml in a suitable Amicon cell, equipped with a 30,000 M. W. cut-off membrane. The concentrate was then charged with 2 volumes of absolute ethanol to precipitate the polysaccharide. This was separated by centrifugation, discarding the supernatant. The pellet was triturated in a small blender with absolute ethanol and the hardened particles collected on a sintered glass funnel. The polysaccharide was washed thereon with 50 ml absolute ethanol and 50 ml acetone and dried in a vacuum desiccator for 24 hours to constant weight. Final Product: 11059-186 (yield 189 mg).

## EXAMPLE 14

## RELEASE PROTOCOL FOR GROUP B STREPTOCOCCUS TYPE II POLYSACCHARIDE, PRODUCT 11059-176

Step 1 - Isolation of First Crude Product

Eighty (80) liters of whole, phenolized broth were charged with 1,170 gm of calcium chloride, dihydrate, to 0.1 M. Ethanol (denatured) was then added to the 40% level (vol/vol). The suspension was allowed to stand for maximal settling of the cells and other insoluble materials (about 48 hours). The

clear supernatant was then pumped over into another fractionation vat, with a recovery of 94 liters. The aqueous phase in this system was calculated as 56.4 liters. On this basis, additional 47 liters of ethanol (denatured) were run in with stirring, to an ethanol level of 60%. Again, the suspension was allowed to stand for maximal settling of the precipitate which contains the desired polysaccharide. The clear supernatant was then aspirated off with the aid of a pump, and the precipitate collected by centrifugation (Beckman, Model J-21C, in 500 ml cups, for 15 min. at 6,000 RPM and at 20°C).

Step 2 - Isolation of Second Crude Product

The precipitate from Step 1 was resuspended in 1% sodium acetate (3,000 ml), giving a final volume of 3,340 ml and the suspension charged with calcium chloride to 0.1 M. Based on a serological probe it could be demonstrated that the desired polysaccharide can be fractionated with ethanol in the range of 38-59%. Accordingly, the suspension was charged with 2,050 ml of ethanol (denatured) to the 38% level. The suspension was cleared by centrifugation, as per above, Step 1. Of the total supernatant recovered (4,850 ml) 3,010 ml constitute the aqueous phase. The supernatant was then charged with ethanol (denatured) (2,470 ml) to 59%. A floculent precipitate developed and settled out readily. It was allowed to settle out and the clear supernatant was removed by aspiration. The precipitate was then collected by centrifugation, as already described.

Step 3 - Removal of Proteinaceous Materials with Trypsin

The slightly pigmented pellet from Step 2 was dissolved in 500 ml 0.05 M sodium borate, pH 8.5 and the solution was charged with 20 mg trypsin (4,760 unit). The system was incubated at 37°C for 90 minutes.

Step 4 - Chromatography on DEAE-Cellulose

The trypsin digest from Step 3 was applied to a column of DEAE-Cellulose (DE-52, Whatman), equilibrated in 0.05 M sodium borate at pH 8.5, (dimension: 5 x 30 cm). The column was then washed with 700 ml borate buffer. The column was then treated with 800 ml of eluant, containing 0.3 M sodium chloride in borate buffer. Effluent fractions (100 ml) were collected and separately assayed serologically for their polysaccharide content, and for precipitability with 1.5 volumes ethanol. Fraction No. 1 did not give a precipitate, Nos. 4 and 5 had the highest amounts of precipitate, and No. 8 gave only a weak precipitate. Fractions No. 2-7 were pooled and the pool charged with 1.5 volumes of ethanol (denatured) to precipitate the polysaccharide.

Step 5 - Isolation of Third Crude Product

The precipitate from Step 4 was taken up in 300 ml distilled water, diafiltered to 100 ml, and the process repeated twice more, in each case with replenishing the volume to 300 ml with distilled water. The final retentate of 100 ml was charged with sodium acetate to 1% and the polysaccharide precipitated with 1.5 volumes of ethanol (denatured). The precipitate was collected by

allowing it to sediment and by decantating of the clear supernatant. The precipitate was then triturated in a blender with ethanol (denatured) and collected on a small sintered glass funnel (15 ml, M), washed thereon with 50 ml ethanol (denatured) and with 50 ml acetone and dried in a vacuum desiccator. Product intermediate: 11059-160 (yield of 2.654 gm).

Step 6 - Cetavlon Complexing of the Polysaccharide

The lot of product 11059-160 was dissolved in 265 ml triply distilled water to give clear, slightly pigmented solution. The solution was then placed in an ice-bath and while stirred, a solution of 2.65 gm Cetavlon in 50 ml of water was added. The resulting suspension was kept at 0°C for 60 minutes. The Cetavlon-polysaccharide complex was then recovered by centrifugation (Sorvall RC-5, in 50 ml cups, at 2°C, 15,000 RPM, for 40 minutes). The supernatant was carefully decanted from a semi-liquid, viscous pellet. The liquid complex was immediately solubilized in 15% sodium acetate (150 ml) at pH 8.4. The clear solution was then charged with ethanol (denatured) to the 60% level, and the precipitate which formed was allowed to settle out. After total decantation of the supernatant, the precipitate was triturated with ethanol in a blender and collected on a small sintered glass funnel (15 ml, M). It was washed thereon with 50 ml of ethanol (denatured), and with 50 ml acetone, followed by drying in a vacuum desiccator. Product Intermediate: 11059-174 (yield 710 mg).

Step 7 - Molecular Sieving on Sepharose 6B and Recovery of Final Product

Four hundred (400) milligrams of product intermediate 11059-174 were dissolved in 12 ml 1% sodium acetate (eluant) and applied to a column of Sepharose 6B (2.6 x 90 cm; equilibrated with 1% sodium acetate). Fractions of 12.5 ml were collected and active fractions were identified by the Ouchterlony method, using type-specific antiserum. Fractions 12-30 were pooled and the pool was reduced in volume to 150 ml in an Amicon stirred cell using YM 30 membrane (having a molecular weigh cut-off of 30,000 daltons). The concentrate was then transferred into a 600-ml beaker and precipitated with two volumes of ethanol (200 proof). The precipitated polysaccharide was allowed o settle out and the clear supernatant removed by decantating. The sticky settlement was triturated in a blender with ethanol and collected on a small sintered glass funnel (15 ml, M) and washed thereon with 50 ml ethanol and 50 ml acetone. It was then dried in a vacuum desiccator to constant weight. Final Product 11059-176 (yield of 285 mg).

EXAMPLE 15

RELEASE PROTOCOL FOR GROUP B STREPTOCOCCUS TYPE III POLYSACCHARIDE, PRODUCT 11059-179

Step 1 - Preparation of Cell-Free Fermentation Broth

Type III broth, phenolized to 1% at termination of the fermentation cycle, was cleared of cells by centrifugation in a Sharples ultracentrifuge, model T-1P.

Step 2 - Ultrafiltration/Diafiltration of Cell-Free Broth

Two hundred (200) liters of the cell-free supernatant from Step 1 were pumped through an ultrafiltration hollow fiber device (Amicon unit, Model DC-2, molecular weight cut-off of 50,000 daltons) to a final volume of 14.9 liters.

Step 3 - Isolation of First Crude Product

The concentrate from Step 2 (14.9 liters) was charged with calcium chloride to 0.1 M and the pH adjusted to 8.2 with 2 N NaOH. It was then charged with 4.95 liters, ethanol (denatured, 2BA T-Type), to 30% by volume. The resulting suspension was centrifuged in a Sharples centrifuge to obtain a clear supernatant (19.0 liters). The aqueous phase of the supernatant was calculated at 14.25 liters. On this basis, the supernatant was charged with additional 21.7 liters ethanol (denatured) to a level of 65%. The resulting suspension was let stand for several days, during which time the desired polysaccharide settled out. The bulk of the clear supernatant was pumped off and the precipitate was collected by centrifugation in 500-ml cups (Beckman, Model J-21C, at 6,000 RPM, 20°C, for 15 minutes). The pellet was triturated in a blender with ethanol (denatured) and collected on a sintered glass funnel (50 ml, M), washed thereon with 50 ml ethanol (denatured) and with 50 ml acetone, followed by drying in a vacuum desiccator overnight. Product 11059-167 (yield 7.22 gm).

Step 4 - Cetavlon Complexing and Recovery of Second Crude Product

Seven (7) grams of product intermediate 11059-167 were dissolved in 700 ml ice-cold water and after stirring for one hour the suspension was cleared by centrifugation. The clear supernatant was then charged with 100 ml of a 7.5% solution of Cetavlon and the resulting suspension allowed to stand at 4°C overnight. The insoluble Cetavlon-polysaccharide complex was collected by centrifugation (Sorvall RC-5, in 50-ml cups, at 15,000 RPM, 4°C for 10 min). The supernatant was decanted from a semi-soft, honey-like pellet. The complex was suspended for extraction in 500 ml 15% sodium acetate (no pH adjustment was needed, since it was noted at 8.2). The suspension was stirred for several hours in the cold to assure maximal solubilization. The centrifugation step was repeated to remove some insoluble, pigmented material. The extracted polysaccharide was precipitated from the extract by the addition of ethanol (denatured) to the 61% level. A sticky gum settled out, allowing the supernatant to be decanted totally.

Step 5 - Removal of Protein by Trypsin Digestion and Recovery of the Third Crude Product

The gum from Step 4 was dissolved in 100 ml of a .3% soluion of sodium acetate, with adjustment of the pH to 8.4. The solution was transferred into a 500-ml centrifuge cup, and charged with 20 mg trypsin. The system was then incubated at 37°C for 90 min. with occasional monitoring of the pH. No further pH adjustments needed to be made. At the

conclusion of the digest cycle, the polysaccharide was recovered by the addition of 2 volumes of ethanol (denatured). The precipitate was collected by centrifugation (Beckman, Model J21C, at 6,000 RPM, at 20°C and for 10 minutes). The pellet was triturated with ethanol (denatured) in a blender, collected on a small sintered glass funnel (15 ml, M), washed thereon with 50 ml ethanol (denatured) and 50 ml acetone, followed by drying in a vacuum desiccator overnight. Product Intermediate 11059-170 (yield 1,357 mg).

Step 6 - Molecular Sieving of Product Intermediate 11059-170

Four hundred (400) milligrams of 11059-170 were dissolved in 12 ml eluant (1% sodium acetate, pH 7.5). The clear solution was then applied to a column of Sepharose 6B, equilibrated with 1% sodium acetate, pH 7.5, (dimensions of 2.6 x 90 cm). Fractions of 12.5 ml were collected and their polysaccharide content monitored serologically by the Ouchterlony double immunodiffusion technique. Active fractions (15-32) were pooled and concentrated to ca. 70 ml (Amicon stirred cell, equipped with a 30,000 M. W. cut-off membrane).

Step 7 - Recovery of Final Product

The concentrated pool from Step 6 was then charged with two volumes of 200 proof ethanol. The gummy precipitate which settled out was removed from the precipitation flask and triturated with absolute ethanol in a blender, collected on a small sintered glass funnel (15 ml, M), washed thereon with 50 ml

absolute ethanol and 50 ml acetone, followed by drying in a vacuum desiccator to constant weight. Product 11059-179 (yield of 272 mg).

WHAT IS CLAIMED IS:

1. A vaccine for Group B Streptococcus infections among neonates or infants and mastitis among dairy mammals comprising a unit dosage or multidose integer of the dosage of an effective amount of at least one of the Group B Streptococcus type-specific polysaccharides selected from a group consisting of

(a) type $I_a$-specific polysaccharide which comprises hexoses, glucosamine and sialic acid in the approximate molar ratio 3:1:1 wherein the hexoses are galactose and glucose, having a molecular weight of about $0.8 \times 10^6$ daltons;

(b) type $I_b$-specific polysaccharide which comprises hexoses, glucosamine, and sialic acid in the approximate molar ratio 3:1:1 wherein the hexoses are galactose and glucose, having a molecular weight of about $0.5 \times 10^6$ daltons;

(c) type II-specific polysaccharide which comprises hexoses, glucosamine and sialic acid in the approximate molar ratio 5:1:1 wherein the hexoses are galactose and glucose, having a molecular weight of about $0.5 \times 10^6$ daltons; and

(d) type III-specific polysaccharide which comprises hexoses, glucosamine, and sialic acid in the approximate molar ratio 3:1:1

wherein the hexoses are galactose and glucose, having a molecular weight of about $0.15 \times 10^6$ daltons;

in a physiologically acceptable medium.

2. The vaccine of Claim 1 comprising at least two of the Group B Streptococcus type-specific polysaccharide types $I_a$, $I_b$, II and III in a physiologically acceptable medium.

3. The vaccine of Claim 1 comprising Group B Streptococcus type-specific polysaccharide types $I_a$, $I_b$, II and III in a physiologically acceptable medium.

4. The Group B Streptococcus type $I_a$ polysaccharide of Claim 1.

5. The Group B Streptococcus type $I_b$ polysaccharide of Claim 1.

6. The Group B Streptococcus type II polysaccharide of Claim 1.

7. The Group B Streptococcus type III polysaccharide of Claim 1.

8. A process for the preparation of an antigenic type-specific polysaccharide of Group B Streptococcus comprising

(a) growing the Group B Streptococcus bacteria types $I_a$, $I_b$, II, or III in a high

glucose, soy bean and yeast extract fermentation medium;

(b) separating the cell paste from the liquid medium;

(c) treating the liquid medium from step b with a strong ionic salt and a water-miscible lower alkanol to precipitate impurities or alternatively digesting the cell paste with an enzyme to obtain a liquid extract;

(d) precipitating the crude polysaccharide from the treated liquid medium, or if desired, the liquid extract from digested cell paste with a sufficient amount of a water-miscible lower alkanol;

(e) suspending the crude polysaccharide in deionized water and adding thereto sufficient cationic detergent to precipitate the polysaccharide;

(f) redissolving the polysaccharide in 15% (wt/wt) sodium acetate aqueous solution;

(g) precipitating the semi-purified polysaccharide from the solution with alcohol and, optionally, digesting the resulting precipitate with a proteolytic enzyme, followed by precipitating the enzyme-treated polysaccharide with a sufficient amount of water-miscible alkanol; and

(h) eluting the resolubilized semi-purified polysaccharide of step g in a liquid medium through a gel column to obtain the purified polysaccharide.

9. The process of Claim 8 wherein the fermentation medium contains sodium chloride, potassium phosphate dibasic, 2.5% by weight glucose, and the dialyzable constituents of soy bean and yeast extracts.

10. A method of passive vaccination of a host against Group B Streptococcus infections comprising injection of an effective amount of antisera to a host with impaired immune system, said antisera being obtained from a donor host who has previously been injected with a vaccine according to Claim 1.

European Patent Office

# PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

0038265
Application number
EP 81 40 0585

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim |
|---|---|---|
| X | EP - A - 0 008 969 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE)<br>* Page 2, line 8 to page 8, line 24 *<br>-- | 1- 9 |
| P | CHEMICAL ABSTRACTS, vol. 93, no. 19, November 10, 1980 page 476, abstract 184091e Columbus. Ohio, USA H.J. JENNINGS et al. "Structure and serology of the native polysaccharide antigen of type Ia group B Streptococcus"<br>& PROC. NATL. ACAD. SCI U.S.A. 1980, 77(5), 2931-5<br>* Whole abstract *<br>-- | 1-9 |
| P | CHEMICAL ABSTRACTS, vol. 92, no. 23, June 9, 1980, page 500, abstract 196165 Columbus, Ohio, USA H.J. JENNINGS et al. "Structural determination and serology of the native polysaccharide antigen of type-III group B Streptococcus"<br>./. | 1-9 |

CLASSIFICATION OF THE APPLICATION (Int. Cl.³)

A 61 K 39/09
C 12 P 19/26

TECHNICAL FIELDS SEARCHED (Int.Cl.³)

A 61 K 39/09

## INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: 1-9
Claims searched incompletely:
Claims not searched: 10
Reason for the limitation of the search:

Method for treatment of the human or animal body by surgery or therapy (See Art. 52(4) of the European Patent Office)

CATEGORY OF CITED DOCUMENTS

- X: particularly relevant
- A: technological background
- O: non-written disclosure
- P: intermediate document
- T: theory or principle underlying the invention
- E: conflicting application
- D: document cited in the application
- L: citation for other reasons

&: member of the same patent family, corresponding document

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| The Hague | 21-07-1981 | REMPP |

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | CLASSIFICATION OF THE APPLICATION (Int. Cl.³) |
|---|---|---|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | |
| | & CAN. J. BIOCHEM. 1980, 58(2), 112-20<br>* Abstract *<br>-- | | |
| P | CHEMICAL ABSTRACTS, vol. 94, no.9, March 2, 1981, page 541, abstract 63452k Columbus, Ohio, USA T.I. DORAN et al. "Extracellular antigens of serotype III group B streptococci"<br>& INFECT. IMMUN, 1980, 30(3), 890-3.<br>* Whole abstract *<br>-- | 1-9 | TECHNICAL FIELDS SEARCHED (Int. Cl.³) |
| | CHEMICAL ABSTRACTS, vol. 90, no. 15, April 9, 1979, page 471, abstract 119515v Columbus, Ohio, USA J.Y. TAI et al. "Isolation of type-specific polysaccharide antigen from group B type Ib streptococci"<br>& J. EXP. MED. 1979, 148(1), 58-66<br>* Whole abstract *<br>-- | 1-9 | |
| | CHEMICAL ABSTRACTS, vol. 93, no. 7, August 18, 1980, page 700, abstract 68332k Columbus, Ohio, USA J.Y. TAI et al. "Isolation of type-specific polysaccharide antigen from group-B type-Ib streptococci" | 1-9 | |

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| DOCUMENTS CONSIDERED TO BE RELEVANT | | | CLASSIFICATION OF THE APPLICATION (Int. Cl.³) |
|---|---|---|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | |
| | & PATHOG. STREPTOCOCCI,PROC. INT. SYMP. 7th 1978, (publ. 1979) , 154-5.<br>* Whole abstract *<br><br>----- | | TECHNICAL FIELDS SEARCHED (Int. Cl.³) |

# United States Patent [19]

**Nimrod et al.**

[11] **Patent Number: 4,780,414**

[45] **Date of Patent: Oct. 25, 1988**


[54] **METHOD OF PRODUCING HIGH MOLECULAR WEIGHT SODIUM HYALLRONATE BY FERMENTATION OF STREPTOCOCCUS**


[75] Inventors: **Abraham Nimrod; Benjamin Greenman; Dov Kanner,** all of Rehovot; **Moshe Landsberg,** Petah Tikva; **Yaffa Beck,** Gedera, all of Israel

[73] Assignee: **Bio-Technology General Corp.,** New York, N.Y.



[21] Appl. No.: **815,957**

[22] Filed: **Jan. 9, 1986**

**Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 692,692, Jan. 18, 1985.

[51] **Int. Cl.**[4] ..... **C12P 19/04;** C12N 1/20; C12R 1/46

[52] **U.S. Cl.** ..... **435/101;** 435/253; 435/803; 435/818; 435/885

[58] **Field of Search** ..... 435/701, 253, 801, 803, 435/818, 885; 536/55.1, 123; 514/54, 62, 847, 915



[56] **References Cited**

U.S. PATENT DOCUMENTS

4,517,295 5/1985 Bracke et al. ..... 435/885

FOREIGN PATENT DOCUMENTS

0037001 3/1983 Japan ..... 536/55.1

OTHER PUBLICATIONS

Buchanan et al.; 'Recovery of Microorganisms from Synovial and Pleural Fluids of Animals Using Hyperosmolar Media'; *Veterinary Microbiology,* vol. 7 (1982); pp. 19–33.

Woolcock, J. B.; 'The Capsule of *Streptococcus equi*', *J. of Gen. Microbial,* vol. 85 (1974); pp. 372–375.

Holmstrom B. et al.; 'Production of Hyaluronic Acid by a Streptococcal Strain in Batch Culture'; *Appl. Microbial.,* vol. 15, No. 6, (1967) pp. 1409–1413.



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[57] **ABSTRACT**

A novel mutant microorganism *Streptococcus zooepidemicus* HA-116 ATCC 39920, has been produced. The microorganism produces large amounts of high molecular weight hyaluronic acid. The invention provides a method of obtaining such microorganisms.

The invention also concerns a method of obtaining sodium hyaluronate which comprises growing with vigorous agitation a microorganism of the genus Streptococcus under appropriate conditions in a suitable nutrient medium containing a sugar component as a carbon source. The sugar component is present in a substantially constant concentration between 0.2 and 10 grams per liter. The medium has a substantially constant pH between about 6.0 and 7.5 and includes a substantially constant magnesium ion concentration above 0.05 grams per liter. The sodium hyaluronate excreted into the medium by the organism is purified using methods involving precipation, redissolving and reprecipating the hyaluronate. Composition of sodium hyaluronate which are characterized by an absence of pyrogenicity and skin irritation are obtained.


**38 Claims, No Drawings**

# METHOD OF PRODUCING HIGH MOLECULAR WEIGHT SODIUM HYALLRONATE BY FERMENTATION OF STREPTOCOCCUS

This application is a continuation-in-part of U.S. Ser. No. 692,692, filed Jan. 18, 1985, the contents of which are hereby incorporated by reference into the present application.

## BACKGROUND OF THE INVENTION

This invention concerns a process for the production of the sodium salt of high molecular weight hyaluronic acid by large-scale fermentation of a microorganism of the genus Streptococcus.

Hyaluronic acid is a naturally occurring glycosaminoglycan consisting of a linear polymer of molecular weight of 50,000–13,000,000 daltons. It is a polysaccharide made of a repeating units of glucuronic acid and N-acetyl-glucosamine, bound by alternating 1–3 and 1–4 bonds.

Hyaluronic acid is present in various connective tissues of animals, such as skin and cartilage. Some organs are specifically rich in hyaluronic acid, such as the umbilical cord, synovial fluid, the vitreous humor and rooster combs. In addition, hyaluronic acid is produced by various microorganisms, such as streptococci Type A and C.

In skin and cartilage, the role of hyaluronic acid is to bind water and retain the tonicity and elasticity of the tissue. In joint fluids, the viscous hyaluronic acid solution serves as a lubricant to provide a protective environment to the cells. A solution of ultrapure hyaluronic acid from rooster combs has been in use for several years as a supportive medium in opthalmic surgery, see U.S. Pat. No. 4,141,973 of E. A. Balazs (1979). A similar preparation has been shown to be beneficial in the treatment of inflamed knee joints of race horses. Another use of hyaluronic acid results from its highly hydrophilic nature, making it an ideal constitutent of moisturization lotions for cosmetic use, U.S. Pat. No. 4,303,676 of E. Balazs (1981).

Hyaluronic acid has been isolated from the various biological sources, as described above, including microbial broth. The isolation and characterization of hyaluronic acid has been described by Meyer et al., J. Biol. Chem. 107,629 (1934); J. Biol. Chem. 114,689 (1936), and has recently been reviewed in Methods in Enzymol. 28, 73 (1972). The structure of hyaluronic acid was elucidated by Weissman et al., J. Am. Chem. Soc. 76, 1753 (1954) and Meyer, Fed. Proc. 17, 1075 (1958).

The production of hyaluronic acid by Streptococcus was first shown by Forrest et al., J. Biol. Chem. 118, 61 (1937), and further elaborated on since by various researchers, such as Roseman et al., J. Biol. Chem 203,213 (1953), Pierce and White, Proc. Soc. Exp. Biol. Med. 87, 50 (1954), U.S. Pat. No. 2,975,104 of G. H. Warren (1961), and Sunghara et al., J. Biol. Chem. 254, 6252 (1979), demonstrating the identity of hyaluronic acid from animal and microbial sources. Procedures have been published for batch fermentations of Type A streptococci and hyaluronic acid isolation on small to medium scales Thonard et al., J. Biol. Chem. 239, 726 (1964); Holmstrom and Ricica, Appl. Microbio. 15, 1409 (1967); Kjems and Lebech, Acta Path. Microbiol. Scand. 84, 162 (1976). These procedures included anaerobic fermentations of the pathogenic bacteria, and resulted in yields of 0.4–1 grams/liter of hyaluronic acid of a molecular weight of 700,000 or less.

Other procedures have concerned the aerobic fermentation of streptococci to produce hyaluronic acid such as Japanese Patent Publication Kokai No. 58-056692, published April 4, 1983, by inventors, Akasaka H, et al. Other publications such as, U.S. Pat. No. 4,141,973, Feb. 27, 1979 by E.A. Balazs, concerned the production and purification of hyaluronic acid from sources such as animal connective tissue. The hyaluronic acid production and purification procedures disclosed in the prior art did not, however, yield hyaluronic acid of an average molecular weight of greater than $2.0\times10^6$ daltons. This is largely due to the fact that hyaluronic acid is easily degraded by shearing or oxidized in reactions catalyzed by impurities or metal ions present in the hyaluronic acid composition.

The novel process described herein results in hyaluronic acid of a molecular weight from about $1\times10^6$ to about $4.0\times10^6$ daltons, in a yield of about 2 grams/liter in anaerobic fermentation and about 4–6 grams/liter in aerobic fermentation. This was made possible by producing a mutant strain of a Type C *Streptococcus zooepidemicus*, HA-116, ATCC 39920, which is a high producer of hyaluronic acid and is haemolysin minue, i.e. of negligible pathogenicity. Aerobic Fermentation of *S. zooepidemicus*, HA-116, ATCC 39920 and subsequent purification of hyaluronate have resulted in batches of sodium hyaluronate with an average molecular weight of greater than $3.5\times10^6$ daltons. This invention is the first method of producing and purifying such high molecular weight sodium hyaluronate by bacterial fermentation.

Hyaluronic acid that is non-pyrogenic and non-irritating can be obtained employing the methods of this invention. Other methods also within the scope of this invention can be employed to produce ultra-pure, non-inflammatory hyaluronic acid suitable for clinical use.

## SUMMARY OF THE INVENTION

The invention concerns a microorganism of the species *Streptococcus zooepidemicus*, HA-116, ATCC 39920, and mutants derived therefrom which are capable of producing sodium hyaluronate by fermentation and excreting it into the surrounding medium.

The invention also concerns a method of obtaining sodium hyaluronate which comprises growing with vigorous agitation a microorganism of the genus Streptococcus under appropriate conditions in a suitable nutrient medium. The medium includes a sugar component as the carbon source in a substantially constant concentration between about 0.2 and 10 grams per liter, has a substantially constant pH between about 6.5 and 7.5 and also includes a substantially constant magnesium ion concentration above 0.05 gram per liter. The microorganism produces sodium hyaluronate and excrete it into the medium. The sodium hyaluronate is then recovered from the medium.

The sodium hyaluronate is recovered from the medium by a method comprising treating in the medium containing the microorganism so as to remove the microorganism and other materials insoluble in the medium, precipitating the sodium hyaluronate from the medium, e.g. precipitation with organic solvents, and recovering the precipitate. The precipitate can then be ground and dried. Compositions of sodium hyaluronate characterized by an absence of pyrogenicity and inflammatory activity can be produced by these methods.

The present invention also concerns a method for selecting microorganisms which produce enhanced amounts of hyaluronic acid and which lack hemolytic activity. The method comprises treating microorganisms that produce hyaluronic acid with a suitable mutagen to produce mutants thereof, and growing the mutants on a suitable solid medium. The mucoid colonies are identified, and recovered. The recovered colonies are grown on blood agar and colonies which do not lyse hemoglobin are selected.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns a method of obtaining high molecular weight sodium hyaluronate from microorganisms of the genus Streptococcus, e.g. S. zooepidemicus or S. equisimilis. The method comprises growing microorganisms of the genus Streptococcus with vigorous agitation under appropriate conditions and in a suitable nutrient medium. The medium includes a sugar component as the carbon source in a substantially constant concentration between about 0.2 and 10 grams per liter, a substantially constant magnesium ion concentration above about 0.05 grams per liter and a substantially constant pH between about 6.5 and 7.5. The microorganisms produce sodium hyaluronate and excrete it into the medium. The sodium hyaluronate is then recovered from the medium.

Any hyaluronic acid producing species of Streptococcus can be used in practicing this invention, e.g. *S. zooepidemicus, S. equisimilis* or *S. pyogenes.* The preferred species is *S. zooepidemicus* and the strain is *S. zooepidemicus* HA-116 ATCC 39920 which is a mutant strain produced according to a method of this invention for obtaining microorganisms which produce an enhanced amount of hyaluronic acid.

The sodium hyaluronate can be obtained by growing the Streptococcus under aerobic or anaerobic conditions. In a preferred embodiment of the invention the appropriate growing conditions comprise aeration of the medium at a rate greater than about 0.5 volumes of air per volume of medium per minute (vvm). An aeration rate of 1–2 vmm is generally used, however greater aeration rates may be desirable. In this preferred embodiment the suitable nutrient medium comprises per liter, casein hydrolysate about 10 to 30 grams, yeast extract about 5 to 15 grams, NaCl about 2 grams, $MgSO_4.7H_2O$ above about 0.5 grams, $K_2HPO_4$ about 2.5 grams, and glucose about 2 to 15 grams.

The sodium hyaluronate can be recovered by treating the medium containing the microorganism so as to remove the microorganism and other materials insoluble in the medium, e.g. by filtration or centrifugation. The sodium hyaluronate is then precipitated from the medium and recovered. The precipitate can then be ground to uniform size particles and dried.

In one embodiment of the invention the sodium hyaluronate is recovered by adjusting the pH of the medium containing the microorganism to a pH of about 5.0 and then heating the medium for a suitable period of time at a temperature between about 80° and 95° C., e.g. heating for 20 minutes at a temperature of about 90° C. or preferably heating for 40 minutes at 80° C. After heating the microorganisms and other insoluble materials are removed. The preferred method of removal is by filtration with a filter aid such as diatomaceous earth.

The sodium hyaluronate can be precipitated from the medium or filtrate by adding a first organic solvent, such as isopropanol, to the medium. The precipitate is redissolved in 3% aqueous sodium acetate and then reprecipitated with a second organic solvent such as ethanol. The second precipitate is redissolved in 3% aqueous sodium acetate and activated charcoal is added to form a suspension. The suspension is filtered and a third organic solvent e.g. acetone is added to produce a precipitate of sodium hyaluronate. The first, second and third organic solvent can each be isopropanol, ethanol or acetone. Alternatively the hyaluronate can be precipated by the same organic solvent in each step, e.g. sodium hyaluronate is precipitated from the medium by using isopropanol in all three of the precipitation steps.

In another embodiment of the invention the pH of the medium containing the microorganism is adjusted to about 7.0 and the medium is cooled to a temperature between about 4° C. and 15° C. and preferably between about 4° and 20° C., prior to treating the medium to remove the microorganism. The medium is then diluted with 3% aqueous sodium acetate to the extent necessary to permit subsequent treatment e.g. three to four-fold.

In one embodiment of the invention, the sodium hyaluronate precipitate is redissolved in 0.15M aqueous NaCl and cetyl-pyridinium chloride is added to form the cetyl-pyridinium salt of hyaluronic acid. The cetyl-pyridinium salt is dissolved in aqueous NaCl and 15% ethanol, e.g. at least 1M NaCl and sodium hyaluronate is recovered therefrom by addition of organic solvent e.g. ethanol precipitating the sodium hyaluronate.

This sodium hyaluronate precipitate can be redissolved in 0.15M aqueous NaCl. Cetyl-pyridinium chloride is added to again form the cetyl-pyridinum salt of hyaluronic acid. The hyaluronic acid salt is dissolved in NaCl (at least about 1M) and ethanol and the sodium hyaluronate is recovered by addition of organic solvent. The precipitate is thereafter dissolved in sterile aqueous 1M NaCl and the resulting solution is contacted with a magnesium silicate absorbant, e.g. Florisil, to remove impurities and residual cetyl-pyridinium ions. The solution is then sterilized and sodium hyaluronate is precipitated by the addition of sterile organic solvent, e.g. sterile isopropanol. The sodium hyaluronate so produced can be air dried under sterile conditions.

In another embodiment of the invention, the medium after fermentation and heating but before microorganisms removal is treated with a first organic solvent. The sodium hyaluronate thus precipitated is recovered, redissolved in 3% aqueous sodium acetate and then activated charcoal is added to form a suspension. The suspension is filtered with a filter aid such as diatomaceous earth and an organic solvent is added to produce a precipitate of sodium hyaluronate. This precipitate is then ground and dried.

In another embodiment of the invention the pH of the medium containing the microorganism is adjusted to about 7.0 and the medium is cooled to a temperature between about 4° and 20° C. prior to treating the medium.

In a preferred embodiment of the invention, an organic solvent, such as ehtanol, is added to the medium containing the microorganisms and the precipitate is collected and washed thoroughly with the organic solvent. The sodium hyaluronate precipitate is redissolved in 0.15M aqueous NaCl and activated charcoal is added. The resulting suspension is filtered with a diatomaceous earth filter aid to remove the charcoal, microorganisms and other insoluble materials. The clear filtrate is then treated with cetyl-pyridinium chloride to form the in-

soluble cetyl-pyridinium salt of hyaluronic acid. The cetyl-pyridinium salt is collected and dissolved in aqueous NaCl containing 10% (v/v) ethanol, e.g. at least 1M NaCl, and sodium hyaluronate is recovered therefrom by addition of organic solvent, e.g. ethanol.

This sodium hyaluronate precipitate can be redissolved in 0.15M aqueous NaCl. Cetyl-pyridinium chloride is added to again form the cetyl-pyridinium salt of hyaluronic acid. The hyaluronic acid salt is dissolved in NaCl (at least about 1M) with 10% ethanol and the dosium hyaluronate is recovered by addition of organic solvent. The precipitate is thereafter dissolved in sterile aqueous 1M NaCl and the resulting solution is contacted with a magnesium silicate absorbant, e.g. Florisil to remove impurities and residual cetyl-pyridinium ions. The solution is then sterilized by filtration and sodium hyaluronate is precipitated by the addition of sterile organic solvent, e.g. sterile ethanol. The sodium hyaluronate so produced can be air dried under sterile conditions.

The sodium hyaluronate is suitable for use in compositions of cosmetic grade and clinical grade sodium hyaluronate and other suitable carriers, e.g. glycerol, polypropylene glycol, sorbitol, collagen, polyethylene glycol.

The cosmetic grade composition of sodium hyaluronate produced by the methods of this invention is characterized by an absence of skin irritation. It contains between about 87% and 91% sodium hyaluronate of a molecular weight between about 700,000 and 1,500,000 daltons and a ratio of glucoronic acid to N-acetyl glucosamine of 1:1, from about 8% to about 12% by weight water, from about 4% to about 5% by weight sodium ion, less than about 0.1% by weight protein, less than about 0.05% by weight sulfate, and less than about 0.5% by weight nucleic acid.

The clinical grade composition of sodium hyaluronate of this invention is characterized by an absence of pyrogenicity and inflammatory activity. It contains between about 88% and 92% by weight sodium hyaluronate of an average molecular weight from about 2 to about $3.5\times10^6$ daltons and a glucuronic acid to N-acetyl glucosamine ratio of 1:1, from about 8% to about 12% by weight water, from about 4% to about 6% by weight sodium ion, less that 0.01% by weight protein, less than 0.001% by weight sulfate, less than 0.02% by weight nucleic acid and less than 0.2% by weight neutral sugar.

A preferred ultrapure composition of sodium hyaluronate produced by the methods of this ivention is characterized by a minimum limiting viscosity of about 3.5m3/kg, a minimum average molecular weight of about $3.5\times10^6$ daltons, a specific optical rotation measured at 25° C. and at a wavelength of 436nm from about 155° to 165°, a protein content of less than about 1mg/gram, an absorbance at the wavelength of 257nm of less than about 0.5, endotoxin of less than about 0.05ng/ml., less than about 0.2mg/g of iron, less than about 0.2mg/g of copper, and an infiltration of less than about 200 white blood cells per $mm^3$ of aqueous humor of owl monkey eye when 1 ml of a 1% solution of the composition dissolved in physiological buffer is implanted in the vitreous replacing about one-half the existing liquid vitreous.

Compositions of high molecular weight sodium hyaluronate of a average molecular weight greater than $3.5\times10^6$ daltons and of different grades of purity have also been produced by the methods of this invention.

The vitreous test in the Owl Monkey Eye was performed essentially as described in U.S. Pat. No. 4,141,973 of E. A. Balazs (1979).

The invention also concerns the microorganism *Streptococcus zooepidemicus* HA-116 ATCC No. 39920 or mutants derived therefrom. This microorganism was derived by a method of selecting microorganisms which produce an enhanced amout of hyaluronic acid and which lack hemolytic activity. The method comprises treating microorganisms that produce hyaluronic acid, such as microorganisms of the genus Streptococcus, with a suitable mutagen capable of producing mutants of the organism, e.g. nitrosoguanidine. The mutants are grown on a suitable solid medium, e.g. Todd-Hewit agar, and mucoid colonies are identified. These colonies are recovered from the solid medium and grown on blood agar. The colonies which do not lyse hemoglobin are then selected and used for the production of hyaluronic acid in accordance with the methods of this invention.

## EXPERIMENTAL DETAILS

## BACTERIA SELECTION AND MUTATION

### Nitrosoguanidine Mutagenesis

Bacteria of the genus Streptococcus were treated for 40 min with 100 mg/ml of N-methyl-N'-nitro-N- nitrosoguanidine in Tris-maleic buffer, pH 6.0, and then allowed to segregate on Todd-Hewitt agar plates for selection of high producers or on blood agar plates for Hemolysin minus selection. The survival rate of the bacteria was usually about 0.1%. Various Type C *Streptococci* obtained from hospital collections were treated as described above.

### Selection for High Production

Visual evaluation of the clones was used for selection of large mucoid colonies. One such colony was obtained from an isolate of a strain which has been typed by the National Streptococcal Reference Center, of the Israeli Ministry of Health, as a variant of Type C *Streptococcus equisimilis* (designated as HA-100). Subsequent tests based on "API 20 Strep" tests (API SYSTEM, S.A. FRANCE) for identification of Streptococci strains, indicate that HA-100 is more closely realted to *S. zooepidemicus.*

### Selection for Hemolysin Minus Mutants

Strain HA-100 was subjected to the mutagenis procedure described above and hemolysin minus [hem.(−)] cololies were examined both for hemolysin activity and for hyaluronic acid production in test-tube fermentation. One hem.(−) mutant which was also a high producer of hyaluronic acid was chosen and used for large scale hyaluronic acid production. This mutant was designated HA-116. "API 20 Strep." tests indicate that HA-116 is a strain of *S. zooepidemicus. Streptococcus zooepidemicus* HA-116 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, and has been assigned accession number ATCC 39920.

## THE FERMENTATION PROCESS

In addition to the use of the selected mutant HA-116, we have devised several other unique procedures to

increase the yields and the molecular weight of the hyaluronic acid produced by bacterial fermentation and to shorten fermentation time. This includes (i) maintenance of high levels of magnesium ion concentrations; and (ii) performance of aerobic fermentations with a high rate of aeration and vigorous agitation.

In a preferred embodiment of the invention the composition of the fermentation medium is as follows:

| Component | Concentration (grams/liter) |
|---|---|
| Casein hydrolysate | 20 |
| Yeast extract | 10 |
| NaCl | 2 |
| $MgSO_4.7H_2O$ | 1.5 |
| $K_2HPO_4$ | 2.5 |
| Glucose | 5 |

In a more preferred embodiment of the invention, the concentration in the fermentation medium of $MgSO_4.7H_2O$ is 1.0g/1, the concentration of glucose is 10g/1 and the other components have the same concentrations as above.

The pH of the medium is maintained at about 7.0 by continuous addition of 5N NaOH upon demand of a pH controller. Concomitant addition of an identical volume of 50% (w/v) glucose is preformed by a pump connected in parallel to the controller.

The cultivation of the bacteria can be conducted with or without aeration. In both cases the cultivation is preferably conducted with vigorous agitation. The aeration is preferably at a rate of about 1–2 volume of air per volume of medium per minute. Yields in the non-aerated fermentors are from about 2 to about 3 g/1 of hyaluronic acid with an average M.W from about 1.5 to about $2\times10^6$. Fermentation with aeration yields from about 4 to about 6 g/1 of hyaluronic acid with an average M.W of from about 2.2 to about $3.3\times10^6$. The average M.W is determined based on viscosity measurements known to those of ordinary skill in the art. In both cases, the incubation time is around 12 hours when a 5% (v/v) incoculum of bacteria which has been grown to 2.0–2.5 O.D. units measured at 660 nm is used. At the end of the fermentation, the density of the biomass is equivalent to a turbidity of 8–13 O.D. units.

## ISOLATION AND PURIFICATION OF HYALURONIC ACID

The hyaluronic acid may be purified by three different procedures, Procedure I, II and III.

## PURIFICATION PROCEDURE I

Purification Procedure I can be divided into two stages A and B. Stage A yields a "cosmetic grade" sodium hyaluronate, while Stage B which is a further purification of the cosmetic grade obtained in Stage A yields a high purity, non-inflammatory material suitable for clinical application.

Stage A

This stage comprises the removal of the bacteria and other insoluble materials by filtration, followed by three successive sedimentation by isopropanol and treatment with activated charcoal.

When only cosmetic grade material is prepared, the fermentation broth is heated for 20 minutes at a temperature of about 90° C. and at a pH of about 5.0 prior to filtration. At this time no dilution is necessary. For the preparation of a clinical-grade high molecular weight material, the fermentation broth is cooled with ice to a temperature from about 10° to about 15° C., diluted 3- to 4-fold with 3% sodium acetate, adjusted to a pH of about 7.0 and then subjected to filtration. Diatomaceous-earth type filter-aid, e.g. .5 grams/liter of Celatom FW-14, Eagle-Picker Industries, Inc., Cincinnati, OH, is used in conjunction with a vacuum-type or pressure filter. Sodium hyaluronate is precipitated from the filtrate by addition of 1 volume of isopropanol. The precipitate is redissolved in an equal volume of 3% sodium acetate, and the material precipitated again with isopropanol. The second precipitate is redissolved in 3% sodium acetate, then 1 gram/liter of activated charcoal is added and the mixture is stirred for about 1 hour. This suspension is filtered and the sodium hyaluronate is precipitated by addition of isopropanol, washed with isopropanol and finally ground and air-dried to give a "cosmetic-grade" product.

Stage B

Cosmetic-grade sodium hyaluronate is purified by two successive precipitations of its cetyl-pyridinium salt, followed by adsorption of impurities on a magnesium silicate, e.g., FLORISIL ®, columnn. FLORISIL ® is a registered trademark of Floridin Co., Berkley Springs, W.Va.

Cetyl-Pyridinium Chloride (CPC) Precipitation:

Cosmetic grade material from Stage A is dissolved in 0.15M NaCl to give a hyaluronate solution of a concentration of about 0.25 percent. One volume of 10% CPC is 0.15M NaCl is added to about 8 volumes of the 0.25% hyaluronate solution. The cetyl-pyridinium salt is separated by decantation and centrifugation, washed with 0.15M NaCl and then redissolved in 2M NaCl containing 15% ehtanol, to give a solution of about 0.2% hyaluronate. Hyaluronic acid is sedimented as the sodium salt by addition of 1 volume of isopropanol. The pellet is washed with isopropanol and redissolved in 0.15M NaCl as described above and the CPC precipitation process is repeated. The isopropanolic precipitate obtained from the second CPC precipitation is then redissolved in 1M NaCl for FLORISIL ® treatment.

Florisil Adsorption:

A solution of about 0.25% sodium hyaluronate in pyrogen-free 1M NaCl is passed through a column of 30–60 mesh activated FLORISIL ® e.g. 200 gr Florisil per 10 liter of solution. The solution is then rendered germ-free by filtration through a 0.2 m filter. Sodium hyaluronate is precipitated by filter-sterilized isopropanol (1 volume), followed by washing with sterile analytical grade ethanol. The precipitate is finally dried by a stream of sterile air.

The yield of hyaluronic acid in this procedure is about 60–70%.

## PURIFICATION PROCEDURES II AND III

Alternatively, two independet purification methods may be employed to obtain cosmetic grade and clinical grade sodium hyaluronate. These procedures are preferred procedure for obtaining sodium hyaluronate. Procedure II yields a low molecular weight "cosmetic grade" sodium hyaluronate, and Procedure III yields a high purity, high molecular weight non-inflammatory sodium hyaluronate suitable for clinical application.

### Procedure II

At the conclusion of fermentation, the fermentation broth is heated to about 90° C., then the pH is adjusted to about 5.0 and the medium kept at 80° for 40 minutes.

This step is terminated by adjusting the pH to 7.0 and cooling to about 20° C. This heating process brings about a drop in the molecular weight of the hyaluronate to about $1-1.5\times10^6$ dalton.

The sodium hyaluronate is precipitated from the fermentation mixture by addition of 1.5 volumes of ethanol. The precipitate is further washed with ethanol to eliminate a larger portion of the microorganisms. This crude material is redissolved in aqueous 3% sodium acetate containing 0.1% parahydroxybenzoic acid methyl ester. The volume is adjusted to give about 2–3 grams per liter of hyaluronate. One gram per liter of activated charcoal and 40 grams per liter of a distomaeous earth-type filter-aid, e.g. Celatom FW-14, Eagle-Picker Industries, Inc., Cincinnati, Ohio, are added to the solution and stirred for at least 1 hour. The mixture is then filtered through a filter-aid cake. Sodium hyaluronate is precipitated by addition of 1.5 volumes of ethanol and the precipitate is redissolved in an equal volume of 3% sodium acetate. This solution is filtered through a fine-pore cotton cartridge and then treated with 1.5 volumes of ethanol. The precipitated purified sodium hyaluronate is ground and finally air-dried to give a "cosmetic grade" product.

### Procedure III

In this procedure, the fermentation broth is treated with 1.5 volumes of ethanol immediately following the conclusion of the fermentation. The precipitated sodium hyaluronate is washed with ethanol to get rid of a large portion of the microorganisms and then redissolved in aqueous 0.15M NaCl containing 0.1% parahydroxybenzoic acid methyl ester. The volume is adjusted to give 1–2 grams per liter of hyaluronate. One gram per liter of activated charcoal and 40 grams per liter of Celatom FW-14 are added to the solution and the mixture stirred for 1 hour. The suspension is then filtered through a cake of filter-aid.

### Cetyl-Pyridinium Chloride (CPC) Precipitation

A 10% solution of CPC in 0.15M NaCl is added to the clear hyaluronate solution. The amount of added CPC solution is calculated to give 5 times in weight the amount of hyaluronic acid. The precipitated cetyl-pyridinium salt is separated by decantation and centrifugation, then redissolved in 1M NaCl containing 10% ethanol by volume to give a solution of about 1–2 grams per liter. Sodium hyaluronate is precipitated by addition of 1.5 volumes of ethanol.

After redissolving the precipitate in 0.15M NaCl the CPC precipitation process described in the immediately preceding paragraph is repeated. The ethanolic precipitate obtained after the second CPC process is then taken for the final purification step.

### FLORISIL ® Adsorption

A solution of about 0.1–0.15% sodium hyaluronate in sterile pyrogen-free 1M NaCl is passed through a column of 30–60 mesh activated FLORISIL ® e.g. 20 gr Florisil per liter of solution. The solution is then rendered germ-free by filtration through a 0.2 um filter. Sodium hyaluronate is precipitated by ethanol (1.5 volumes), followed by washing with analytical grade ethanol. The precipitate is finally dried by a stream of sterile nitrogen.

The yield of hyaluronic acid in this procedure is about 70–80%.

## PROPERTIES OF THE PRODUCT SODIUM HYALURONATE

Sodium Hyaluronate Grade I

Sodium hyaluronate grade I is "cosmetic grade" sodium hyaluronate that is obtained after purification Stage A of Procedure I or Procedure II. Its properties are as described below:

a. Content of Sodium Hyaluronate:

87–91%, assayed by the modified carbazole method, Bitter and Muir, Anal. Biochem. 4, 330 (1962) using Sigma hyaluronic acid Type I, cat. #H 1751, as a reference standard.

b. Average Molecular Weight:

From about 700,000 to about 1,500,000 daltons, calculated from the limiting viscosity number esentially as described by Laurent et al., Biochem. Biophys. Acta 42, 476 (1960). A representative calculation of intrinsic viscosity of molecular weight is shown below.

Intrinsic Viscosity of Molecular Weight:

The viscosity of sodium hyaluronate (NaHA) solutions was measured with a capillary viscometer. The flow time (t) of the sample was measured and compared with the flow time (t) of pure solvent.

Viscometer: Cannon-Ubbelohde dilution viscometer size 100 (Cannon Instrument Co.).

Solution: 0.1% sodium hyaluronate in 0.2 M sodium chloride

Temperature: 25° C.+0.01

Calculation of instrinsic viscosity:

$\eta$rel. - Relative viscosity expresses the change in solution viscosity relative to the pure solvent.

$$\eta\text{rel.} - \frac{\eta\text{sample}}{\eta\text{reference}} = \frac{\rho\text{sample} \times t\ \text{sample}}{\rho\text{reference} \times t\ \text{reference}}$$

$\rho$ - density

$t$ - flow time in seconds

$\eta sp$ - Specific viscosity. Measures the increase in viscosity over unity.

$$\eta sp - \frac{t\ \text{sample} \times t\ \text{reference}}{t\ \text{reference}} = ref - 1$$

$\eta sp/C$ - reduced viscosity

$C$ - concentration in $gr$/ml

$(\eta)$ - intrinsic viscosity (limiting viscosity number)

$$(\eta) = C\lim_0 \eta^{sp/C}$$

Determination of intrinsic viscosity ($\eta$):

The viscosity of a 0.1% sodium hyaluronate solution and of two fold, three fold and four fold dilutions of this solution were measured. The concentration of sodium hyaluronate was determined by the carbozole method. sp/C was plotted versus C and extrapolated linearly to C=0. ($\eta$) was obtained from the intersect of the line with the Y-axis.

Determination of molecular weight:

The molecular weight of sodium hyaluronate was calculated from the empirically-established Mark-Houwink relationship

$$(\eta)=0.0403.M^{0.775}$$

wherein M is the molecular weight in daltons. The above relationship was used to determine the molecular weight of various lots of NaHA produced. The relationship is shown in Table I.

TABLE I

| [η], ml/g | Mol. Wt., Dalton |
|---|---|
| 800 | 350,000 |
| 1,200 | 590,000 |
| 1,600 | 860,000 |
| 2,000 | 1,145,000 |
| 2,600 | 1,600,000 |
| 3,200 | 2,100,000 |

c. Ratio of Glucuronic Acid/N-Acetyl Glucosamine (NAG): 1/1; NAG assayed by the modified method of Morgan and Elson, methods in Carbohydrate Chemistry 8, 89 (1980).

d. WATER CONTENT: 10%±2%.

e. Protein: Less than 0.1%, assayed by the Coomasie blue method of Bradford, Anal. Biochem. 72, 248 (1976).

f. Sodium Ions: 5%+1%, assayed by flame photometry.

g. Sulfate Content: Less than 0.05%, as determined after hydrolysis in hydrochloric acid by the turbidometric method of Roden et al., Methods Enzymol. 28, 73 (1972).

h. Nucleic Acids: Less than 0.5%, assayed by measurement of the absorbance of light through a 1% solution at a wavelength of 260 m.

i. Absence of Skin Irritation: This is determined for a 1% solution by (i) Draize dermal irritation test in rabbits, Draize, J.H., in: "Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics". Association of Food and Drug Officials of the United States, Austin, Texas, pp. 46–49 (1959); (ii) Delayed contact hypersensitivity test in Guinea pigs, Magnusson and Kligman, J. Invest. Dermatol. 52, 268 (1969).

Sodium Hyaluronate Grade II

Sodium hyaluronate Grade II is "clinical grade" sodium hyaluronate obtained after purification through Stage B of Procedure I or after purification by Procedure III. Its properties are as described below:

a. Content of Sodium Hyaluronate: 88–92%.

b. Average Molecular Weight: More than $7\times10^5$ daltons, usually in the range from about 2 to about $3.5\times10^6$ daltons for NaHA purified through Stage B of Procedure I and in the range from about 2 to about $4.4\times10^6$ daltons for NaHA purified by Procedure III. These molecular weight ranges are calculated from the limiting viscosity number as described above.

c. Ratio of Glucuronic Acid/N-acetyl Glucosamine: 1/1.

d. Water Content: 10%±2%

e. Protein: Undetectable (less than 0.01%).

f. Sodium Ions: 5%±1%

g. sulfate Content: Undetectable (less than 0.001%).

h. Nucleic Acids: Undetectable (less than 0.02%).

i. Neutral Sugars: Undetectable (less than 0.2%). The neutral sugars are determined in samples after hydrolysis. Hydrolysis is for 1 hour at 120° C. in 2N trifluoroacetic acid followed by vacuum drying. Thin layer chromatography is preformed on silicagel thin layer plates (0.2 mm), pretreated with 0.02 M sodium acetate. Sample hydrolysates are loaded along with reference standards and run in acetone: water, 90:10. The sugar spots are detected by charring with sulfuric acid.

j. Pyrogenicity: Negative. Pyrogenicity is measured by the standard methods known to those of ordinary skill in the art, after injection of a 1% solution in rabbits.

k. Absence of Inflammatory Activity: This property is determined of a sensitive assay method utilizing mice. The method is based on the migration of white blood cells, mainly polymorphonuclear cells and macrophages, into the peritoneum after introduction of an inflammatory agent. These cells are sensitized by the inflammatory process to produce superoxide radicals. The migration and sensitization are assayed by the following procedure: 1 ml samples are injected intraperitoneally, into groups of 2 to 3 mice. 24 hours later the peritoneum of each animal is washed 3 times with 5 ml of Earle's medium. The washes from the mice in each group are combined. Cells are sedimented at 1,500 RPM for 10 min, and resuspended in 1 ml for counting. The volumes of the samples are then adjusted to give $4\times10^6$ *cells/ml, and* 0.25 ml portions are taken for 90 min incubation with 0.5 ml of 2 mg/ml cytochrome C and graded amounts (0, 2, 10, and 20 mg final) of phorbol myristate acetate (PMA). PMA is an activator of the oxidative "burst" system. The media are centrifuged at 1,500 RPM for 15 min and the absorbance of the supernatants is determined at 550 nm.

Inflammation is indicated by an increase in both the number of peritoneal cells and the maximal ability to respond to PMA and reduce the cytochrome C. Hence, an index of inflammation is defined as the activity (in nmoles of superoxide radicals formed) of all the while cells obtained from one mouse. A sample is regarded as non-inflammatory if the inflammation index is not significantly higher than that obtained from mice injected with saline alone.

What is claimed is:

1. The microorganism Streptococcus zooepidemicus HA-116 ATTC No. 39920 and mutants derived therefrom.

2. A method of obtaining sodium hyaluronate which comprises (a) growing a microorganism of the genus Streptococcus under appropriate conditions in a suitable nutrient medium, the conditions comprising vigorous agitation and aeration of the medium and the medium including a sugar component as the carbon source in a substantially constant concentration between about 0.2 and 10 grams per liter, having a substantially constant pH between about 6.5 and 7.5 and including a substantially constant magnesium ion concentration above about 0.05 grams per liter, such that the microorganism produces sodium hyaluronate and excretes the sodium hyaluronate so produced into the medium, and (b) then recovering the sodium hyaluronate from the medium.

3. A method of claim 2, wherein the microorganism is of the species Streptococcus zooepidemicus.

4. A method of claim 3, wherein the microorganism is Streptococcus zooepidemicus, HA-116, ATCC 39920.

5. The method of claim 2, wherein the aeration of the medium is at a rate greater than about 0.5 volume of air per volume of medium per minute.

6. The method of claim 2, wherein the suitable nutrient medium comprises in the concentration of grams per liter of the medium the following components:

| component | concentration |
|---|---|
| Casein hydrolysate | about 10-30 |
| Yeast extract | about 5-15 |
| NaCl | about 2 |
| $MgSO_4.7H_2O$ | above about 0.5 |
| $K_2HPO_4$ | about 2.5 |
| Glucose | about 2-15 |

7. The method of claim 2, wherein recovering the sodium hyaluronate comprises treating the medium containing the microorganisms so as to remove the microorganism and other materials insoluble in the medium, precipitating the sodium hyaluronate from the medium and then recovering the precipitate.

8. The method of claim 7 further comprising grinding and then drying the precipitate.

9. The method of claim 7, further comprising adjusting the pH of the medium containing the microorganism to about 5.0 and then heating the medium for a suitable period of time at a temperature between about 80° and 95° C. prior to treating the medium to remove the microorganism.

10. The method of claim 9, wherein the medium is heated for about 20 minutes at about 90° C.

11. The method of claim 9, wherein the medium is heated for about 40 minutes at about 80° C.

12. The method of claim 7, wherein the treating comprises filtration.

13. The method of claim 12, wherein the filtration comprises filtration on a diatomaceous earth.

14. The method of claim 7, wherein the precipitation comprises adding a first organic solvent to the medium to produce a precipitate, redissolving the precipitate in 3% aqueous sodium acetate, adding a second organic solvent to produce a precipitate, redissolving the precipitate in 3% aqueous sodium acetate, adding activated charcoal to form a suspension, filtering the suspension and adding a third organic solvent to the filtrate to produce a precipitate of sodium hyaluronate.

15. The method of claim 14, wherein each of the first, second and third organic solvents are isopropanol, ethanol or acetone.

16. The method of claim 15, wherein the first, second and third organic solvents are isopropanol.

17. The method of claim 7, further comprising adjusting the pH of the medium containing the microorganism to about 7.0, cooling the medium to a temperature between about 4° and 15° C. and then diluting the medium with 3% aqueous sodium acetate prior to treating the medium to remove the microorganism.

18. The method of claim 7, further comprising adjusting the pH of the medium containing the microorganism to about 7.0, cooling the medium to a temperature between about 4° and 20° C. and then diluting the medium with 3% aqueous sodium acetate prior to treating the medium to remove the microorganism.

19. The method of claim 14, further comprising adjusting the pH of the medium containing the microorganism to about 7.0, cooling the medium to a temperature between about 4° and 15° C. and then diluting the medium with 3% aqueous sodium acetate prior to treating the medium to remove the microorganism.

20. The method of claim 19, further comprising redissolving the precipitate in 0.15M squeous NaCl, adding cetyl-pyridinium chloride to form the cetyl-pyridinium salt of hyaluronic acid, dissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) and ethanol, adding organic solvent and recovering the sodium hyaluronate.

21. The method of claim 20, further comprising redissolving the recovered sodium hyaluronate in 0.15M aqueous NaCl, adding cetyl-pyridinium chloride to again form the cetyl-pyridinium salt of hycluronic acid, dissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) and 10% ethanol, precipitating the sodium hyaluronate with an organic solvent, dissolving the sodium salt in NaCl solution, contacting the resulting solution with magnesium silicate absorbent to remove impurities and residual cetyl-pyridinium ions, sterilizing the solution and adding sterile organic solvent to precipitate the sodium hyaluronate from the solution.

22. The method of claim 21, wherein the organic solvent is isopropanol.

23. The method of claim 21, further comprising aird-rying the sodium hyaluronate precipitate under sterile conditions.

24. The method of claim 2, wherein recovering the sodium hyaluronate comprises adding a first organic solvent to the medium to produce a precipitate, washing the precipitate with more organic solvent, redissolving the precipitate in a suitable aqueous solution, adding activated charcoal to form a suspension and filtering the suspension to remove residual microorganisms and other insoluble materials.

25. The method of claim 24, further comprising adjusting the pH of the medium containing the microorganism to about 5.0 and heating the medium for a suitable period of time at a temperature between about 80° and 95° C. prior to adding the first organic solvent.

26. The method of claim 25, wherein the suitable period of time is 40 minutes and the temperature is 80° C.

27. The method of claim 24, wherein the filtration comprises filtration on a diatomaceous earth.

28. The method of claim 24, wherein the suitable aqueous solution 3% sodium acetate.

29. The method of claim 24, further comprising adding a second organic solvent to the filtrate to produce a precipitate, redissolving the precipitate in 3% aqueous sodium actate, filtering the solution and adding a third organic solvent to the filtrate to produce a precipitate of sodium hyaluronate.

30. The method of claim 29, wherein each of the first, second and third organic solvents are isopropanol, ethanol or acetone.

31. The method of claim 29, wherein the first, second and third organic solvents are ethanol.

32. The method of claim 29, further comprising grinding and then drying the precipitate of the third organic solvent.

33. The method of claim 24, 25 or 29, further comprising adjusting the pH of the medium containing the microorganism to about 7.0 and cooling the medium to a temperature between about 4° and 20° C. prior to adding the first organic solvent.

34. The method of claim 24, wherein the suitable aqueous solution is 0.15M aqueous NaCl solution containing 0.1% parahydrozybenzoic acid methyl ester.

35. The method of claim 34, further comprising adding cetyl-pyridinium chloride in 0.15M NaCl to the hyaluronate solution to form the cetyl-pyridinium salt to hyaluronic acid, redissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) containing 10%

ethanol and adding organic solvent to recover the sodium hyaluronate.

36. The method of claim 35, further comprising redissolving the recovered sodium hyaluronate in 0.15M aqueous NaCl, adding cetyl-pyridinium chloride to again form the cetyl-pyridinium salt of hyaluronic acid, redissolving the cetyl-pyridinium salt in aqueous NaCl (at least about 1M) containing 10% ethanol, adding organic solvent to precipitate the sodium hyaluronate, redissolving the precipitate in sterile 1M aqueous NaCl, contacting the resulting solution with magnesium silicate absorbent to remove impurities and residual cetyl-pyridinium ions, sterilizing the solution, and adding sterile organic solvent to precipitate the sodium hyaluronate from the solution.

37. The method of claim 36, wherein the organic solvent is ethanol.

38. The method of claim 36, further comprising drying the sodium hyaluronate by nitrogen under sterile conditions.

* * * * *

# United States Patent [19]

**Bracke et al.**

[11] **Patent Number: 4,517,295**

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[54] **HYALURONIC ACID FROM BACTERIAL CULTURE**


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[56] **References Cited**

U.S. PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| 2,975,104 | 3/1961 | Warren | 435/101 |
| 4,141,973 | 2/1979 | Balazs | 424/180 |
| 4,303,676 | 12/1981 | Balazs | 424/359 |
| 4,328,803 | 5/1982 | Pape | 424/180 |
| 4,393,136 | 7/1983 | Cheetham | 435/161 |

FOREIGN PATENT DOCUMENTS

| | | |
|---|---|---|
| 183707 | 11/1982 | Japan . |
| 212300 | 12/1982 | Japan . |
| 56692 | 4/1983 | Japan . |
| 57319 | 4/1983 | Japan . |
| 1557755 | 12/1979 | United Kingdom . |
| 950735 | 8/1982 | U.S.S.R. . |

OTHER PUBLICATIONS

Van De Rijn et al., Infection and Immunity, vol. 27, No. 2, pp. 444–448, (Feb. 1980).

Merck Index 9th Edition 1976 entry, 4634—"Hyaluronic Acid".

"Isolation of Hyaluronic Acid from Cultures of Streptococci in a Chemically Defined Medium," *Acta Path. Microbiol. Scand,* Sect. B, 84:162–164, 1976.

Cumulated Index Medicus, 1980 (three pages) Specifically Hyaluronic Acid Entry, p. 1; Biosynthesis, p. 2 and Biosynthesis p. 3.

"The Biosynthesis of Hyaluronate by Group A Streptococci," Yael Gintzburg, a 1955 Thesis at the University of Minnesota.

"The Biosynthesis of Hyaluronic Acid by Group A Streptococcus," *Journal of BioChemistry,* 1953, Roseman, Saul et al., pp. 213, 224, 225.

"The Effect of Diflunisal on Hyaluronic Acid Production by 'Activated' Human Snovial Fibroblasts," *Br. J. Clin. Pharmac.* (1981), 12, 423–426 by Michael Yaron and Ilana Yaron.

"Biosynthesis of Proteo Glycans and Hyaluronate in Human Fibroblast Cultures Established from Normal and Pathological Corneas," *Exp. Eye Res.,* 1981, 32, 435–443, by Inger Marie Dahl.

"Biosynthesis of Polysaccharides by Prokaryotes," *Ann Rev. Microbiol,* 1979, 33:169–199, by Sheri J. Tonn and J. E. Gander.

"Effect of Cycloheximide and Dexamethasome Phosphate on Hyaluronic Acid Synthesis and Secretion in Cultured Human Skin Fibroblasts," *Journal of Cellular Physiology,* 109:215–222, (1981), by J. L. Mapleson and M. Buchwald.

"Hyaluronic Acid and Hyaluronaise Produced by 78 Hemolytic Streptococci, Comprising 40 Types of Group A[1] (Table 1)," *Acta. Pathol. Micro. Scand.* 35, (1954), 159–164, by V. Faber and K. Rosendal.

"The Biosynthesis of Hyaluronic Acid," *Chem. Mol. Biol. of the Intercell,* Matrix 2, E. A. Balazs (Ed.) 1970, by Bernard Jacobson, pp. 763–781.

"The Biosynthesis of Hyaluronic Acid in Group A Streptococci," *Chem. Mol. Biol. of the Intercell,* Matrix 2, pp. 783–794, E. A. Balazs (Ed.) 1970, by Stoolmiller and Dorfman.

"Type IIA Hyperlipoproteinemic Sera Decrease the Synthesis of Hyaluronic Acid by Cultured Human Aortic Smooth Muscle Cells," *Atherosclerosis,* 39, (1981), 61–69, by Jarvelainen, Ronnemaa et al.

"The Synovial Fluid Hyaluronic Acid in Rheumatoid Arthritis", *Experimentia,* 34 (12): p. 1545, (1978), by J. A. Kofoed.

"Abnormalities of Connective Tissue Cells Cultured from Patients with Rheumatoid Arthritis" from Department of Internal Medicine, and the Rackham Arthritis Research Unit, The University of Michigan Medical School, Ann Arbor, Michigan, pp. 65–75.

"Preparation and Stability of Hyaluronic Acid" *Biochem. Biophys. Acta.,* 53, (1961), 254–262, by Ward Pigman et al.

"Studies on Hyaluronic Acid I. The Preparation and Properties of Rooster Comb Hyaluronic Acid," *Biochimica et Biophysica Acta,* 156, (1968), 17–30, by David A. Swann.

"The Hydrolysis of the Polysaccharide Acids of Vitreous Humor, of Umbilical Cord, and of Streptociccus of the Autolytic Enzyme of Pneumococcus", J.B.C. 118, (1937), pp. 71–78, by Karl Meyer.



(List continued on next page.)



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*Attorney, Agent, or Firm*—Schroeder, Siegfried, Vidas & Arrett



[57] **ABSTRACT**

Hyaluronic acid, a polysaccharide, is prepared in high yield from streptococcus bacteria by fermenting the bacteria under anaerobic conditions in a $CO_2$-enriched growth medium, separating the bacteria from the resulting broth and isolating the hyaluronic acid from the remaining constituents of the broth. The bacteria may be grown free of endotoxins by filtering all ingredients through a 10K Millipore ® filter prior to inoculation of the medium and subsequently maintaining pyrogen-free conditions. Separation of the microorganisms from the polysaccharide is facilitated by killing the bacteria with trichloroacetic acid. After removal of the bacterial cells and concentration of the higher molecular weight fermentation products, the hyaluronic acid is isolated and purified by precipitation, resuspension and reprecipitation.


**30 Claims, No Drawings**

OTHER PUBLICATIONS

Abstract Entitled, "Hyaluronic Acid Capsule; Strategy for Oxygen Resistance in Group A Streptococci," *Chemical Abstracts,* vol. 92, Abstract 90592w, 1980, p. 274 by Cleary P. Patrick.

Abstract entitled, "The Mechanism of Estrogen-Induced Increase in Hyaluronic Acid Biosynthesis, with Special Reference to Estrogen Receptor in the Mouse Skin," *Chemical Abstracts,* vol. 92, Abstract 88269c, 1980, by Makoto Usuka, pp. 63, 64.

Abstract entitled, "Effects of Sodium Aurothiomalate on Hyaluronic Acid Synthesis in Normal and Rheumatoid Synovial Fibroblast Cultures," *Chemical Abstracts,* vol. 92, 1980, by Eero Vuorio et al., p. 54, Abstract 33945q.

"Cell-Free Synthesis of Hyaluronic Acid in Marfan Syndrome," *Journal of Biological Chemistry,* (1979), pp. 12199-12203.

"Biosynthesis of Proteoglycans and Hyaluronate in Rabbit Corneal Fibroblast Cultures, Variation with Age of the Cell Line and Effect of Foetal Calf Serum," *Exp. Eye Res.,* (1981) 32, 419-423, by Inger Marie Dahl.

"The Role of Glutamine in the Biosynthesis of Hyaluronate by Streptococcal Suspensions," *Biochemical Journal,* vol. 62, (1956), by D. A. Lowther et al., pp. 304-314.

"The Capsulation of Streptococci and its Relation to Diffusion Factor (Hyaluronidase)", *Journal of Pathology and Bacteriology,* vol. 53, (1941), pp. 13-27, by Douglas McClean.

"Hyaluronate Capsule Prevents Attachment of Group A Streptococci to Mouse Peritoneal Macrophages," *Infection and Immunity,* Mar. 1981, pp. 985-991, by Ellen Whitnack et al.

"The Biosynthesis of Hyaluronic Acid by Group A Streptococcus VI. Biosynthesis from Uridine Nucleotides in Cell-Free Extracts," *The Journal of Biological Chemistry,* vol. 234, No. 9, pp. 2343 and 2350, Sep. 1959, by Alvin Markovitz et al.

"The Biosynthesis of Hyaluronic Acid by Group A Streptococcus V. The Uridine Nucleotides of Group A Streptococcus," *The Journal of Biological Chemistry,* (1957), 228: 547-557, by J. A. Cifonelli.

"Hyaluronic Acid—Chapter 2," *Carbohydrates of Living Tissue,* by M. Stacey et al., (1962), pp. 37-58.

"A Method for the Purification of Bovine Vitreous Body Hyaluronic Acid" *Biochimica et Biophysica Acta* 673, (1981), 192-196, by Otto Schmut et al.

"Structure of Hyaluronic Acid," *Chem. & Mol. Biol. of the Intercellular* Matrix 2, E. A. Balazs (1970) by T. C. Laurent, pp. 703-732.

"Biosynthesis of Hyaluronic Acid by Streptococcus," *The Journal of Biological Chemistry,* vol. 254, No. 14, issued Jul. 25, 1979, pp. 6252-6261, by Sugahara et al.

"The Isolation and Characterization of Hyaluronic Acid from Pasteurella Multocida," *Carbohydrate Research,* 14 (1970), 272-276 by J. A. Cifonelli.

"Streptococcal Bacteriophage 12/12-Borne Hyaluronidase and its Characterization as a Lyase (EC 4.2.99.1) by Means of Streptococcal Hyaluronic Acid and Purified Bacteriophage Suspensions," *Acta Path. Microbiol. Scand.* Sect. B, 84: 145-153, 1976 by H. Niemann et al.

"A Serologically Inactive Polysaccharide elaborated by Mucoid Strains of Group A Hemolytic Streptociccus," *Journal of Biological Chemistry,* (1937), pp. 61, 68, and 69, by Forrest E. Kendall et al.

"III. Hyaluronic Acid," *Mucopolysaccharides of Higher Animals in the Carbohydrates,* vol. IIB. (1970), 2nd Ed. by R. W. Jeanloz, pp, 592-597.

"Sequential Hydrolysis of Hyaluronate by B--glucuronidas and B-N-acetylhexosaminidase," *Biochem. J.* (1981) 197, 275-282, by Maria O. Longas et al.

"Comparison of Media and Culture Techniques for Detection of Streptococcus Pneumoniae in Respiratory Secretions," *Journal of Clinical Microbiology,* Dec. 1980, pp. 772-775 by T. C. Wu et al.

"Hyaluronic Acid Capsule: Strategy for Oxygen Resistance in Group A Streptococci," *Journal of Bacteriology,* Dec. 1979, pp. 1090-1097 by P. Patrick Cleary.

"Carbon Dioxide Control of Lag Period and Growth of Streptococcus Sanguis," *Journal of Bacteriology,* vol. 117, No. 2, Feb. 1974, pp. 652-659 by Roy Repaske et al.

"The Biosynthesis of Hyaluronic Acid by Streptococcus," *The Journal of Biological Chemistry,* vol. 244, No. 2, Jan. 25, 1969, pp. 236-246 by A. C. Stoolmiller et al.

"Hyaluronic Acid in Plants of Microorganisms," bibliography prepared by *Bibliographic Retrieval Services, Inc.* (dated 4/03/82).

"Hyaluronidase Bibliography" by *Worthington Biochemical Corporation,* Freehold, N.J. (through 1970).

"Rapid Purification of Interferon with Millipore Pellicon Cassette Systems, *Technical Service Brief,* Millipore Corporation, Bedford, Mass., Cat. No. TS062, 1981.

"Pellicon Cassette System—Assembly, Operation, Maintenance Instructions," Millipore Corporation, Bedford, Mass., (brochure) Cat. No. OM029, Part No. 11041, 3/1981.

# HYALURONIC ACID FROM BACTERIAL CULTURE

## BACKGROUND OF THE INVENTION

Hyaluronic acid is a mucoid polysaccharide of biological origin. The sodium salt, sodium hyaluronate, in buffered physiological saline solution, has found significant use as a vitreous replacement in optical surgery and in other medical applications. Some of these applications are described in U.S. Pat. Nos. 4,141,973 and 4,328,803. For such medical purposes, a pyrogen-free, highly purified sodium hyaluronate having a molecular weight in excess of 750,000 has heretofore been used. A commercial product known as HEALON TM, manufactured by Pharmacia, Inc., Piscataway, N.J., is a one-percent solution of sodium hyaluronate sold for such purposes. For example, dilute HEALON TM solution (0.1–0.2% sodium hyaluronate) has been reported to be useful as an eye drop for the treatment of patients with keratitis sicca syndrome.

Hyaluronic acid has also been used as an ingredient for in vitro culture of leprosy baccili and as a component for cosmetic formulations. Cosmetic formulations, which are described in U.S. Pat. No. 4,303,676, include both a low molecular weight fraction (about 10–50,000) and a higher molecular weight fraction (in excess of $1 \times 10^6$).

The source of hyaluronic acid for all of the foregoing uses have been rooster combs, human umbilical cords or other vertebrate tissue. Extraction and purification of hyaluronic acid from such tissue is a relatively complex process which results in a very expensive product.

Hyaluronic acid can be produced by Group A and C strains of Streptococcus bacteria. One use reported for the bacterial product appears to be as a reagent for determination of anti-streptococcal hyaluronidase in human serum samples, Kjems and Lebech, Acta Path. microbiol scand., Section B, 84: 162–164 (1976). In that paper, the authors describe a defined media for growing Group A streptococci and isolating hyaluronic acid, reporting a yield of 0.3 grams per liter of culture broth. However, hyaluronic acid produced by bacteria has not found substantial use because it is of a low molecular weight range.

## BRIEF DESCRIPTION OF THE INVENTION

The present invention provides hyaluronic acid from bacterial sources of a preferred higher molecular weight range than is reported in the prior art as being obtained from bacterial sources. It also provides a method for producing hyaluronic acid from bacterial sources in much higher yields than has previously been reported. The method additionally produces hyaluronic acid which has a purity comparable to or better than any presently available for medical applications. Although the hyaluronic acid produced by the present method typically has an average molecular weight of about 55,000, it has potentially significant use as an eye drop ingredient and as an ingredient of cosmetic formulations. The high yield, high purity and low cost of the hyaluronic acid produced by the inventive method also permits it to be used in ways not previously described or contemplated for hyaluronic acid obtained from mammalian or low yield bacterial sources. For instance, hyaluronic acid might be used in food preparations as a humectant, in other applications as a lubricant, and in post-surgical applications for reducing complications due to fibrotic response and/or adhesion formation. The material might also be used in tertiary oil recovery as a substitute for polyacrylamide, similar synthetic polymers or biologically-produced polymers.

The inventive method in its preferred form comprises growing a culture of a hyaluronic acid-producing streptococcus strain under anaerobic conditions in a $CO_2$-enriched growth medium, which includes those raw materials necessary for the production of the hyaluronic acid by the bacteria, preferably although not necessarily killing the bacteria, separating the bacteria from the growth medium and isolating the hyaluronic acid. Preferably, growth is accomplished by fermentation in a broth culture. Other growth techniques and media may be used. For example, an agar culture may be used. Hence, the term "growth medium" herein is to be taken broadly as meaning liquid or solid media or combinations thereof and other types of growth in addition to fermentation, as are all well known in the art.

Although the preferred form of the invention contemplates the growth of bacteria directly in the culture medium in which the hyaluronic acid is to be produced, it is also possible to grow the bacteria in other growth media, separate the bacteria from the medium, resuspend the bacteria in a buffered suspension medium or distilled water and add the appropriate raw materials to the suspension for the production of the hyaluronic acid by the already grown bacteria. This is considered to be an art equivalent to the preferred method and merely involves the use of a resting cell suspension. Consequently, such terminology herein as "growing a culture" and the like is to be taken as including both approaches within its purview.

Unlike prior methods of hyaluronic acid production, endotoxins can be excluded from the system initially by filtering all ingredients through a 10,000 (10K) nominal molecular weight limit (NMWL) cutoff filter, such as the Millipore® Pellicon® cassette tangential flow filtration system, prior to inoculation and subsequently maintaining pyrogen-free anaerobic growth conditions.

## DETAILED DESCRIPTION OF THE INVENTION

In the preferred embodiment of the present invention, a semi-defined growth medium such as the following is used:

1. Casein hydrolysate, enzymatic: 20.0 g
2. Potassium chloride: 3.0 g
3. Sodium phosphate, dibasic: 2.8 g
4. Magnesium sulfate ($7H_2O$): 0.5 g
5. Calcium chloride ($2H_2O$): 10.0 mg
6. Glucose: 20.0 g
7. Vitamin solutions:
   - (a) d-Biotin: 1.0 mg
   - (b) D-Calcium Pantothenate: 1.0 mg
   - (c) Choline chloride: 1.0 mg
   - (d) Folic acid: 1.0 mg
   - (e) i-Inositol: 2.0 mg
   - (f) Nicotinamide: 1.0 mg
   - (g) Pyridoxal HCl: 1.0 mg
   - (h) Riboflavin: 0.1 mg
   - (i) Thiamine HCl: 1.0 mg

The medium is made up to one liter with reverse osmosis pyrogen-free water. Of course, other growth media suitable for this purpose may also be used.

A 100 ml culture of *Streptococcus pyogenes* type is grown anaerobically for six hours in the medium at

37±1° C. The other hyaluronic acid-producing bacteria referred to hereinabove may be used, but the type 18 is preferred. Five liters of the same medium are inoculated with this six-hour culture and grown to a high visible density, preferably to at least $2\times10^8$ cells per ml and typically to $5\times10^8$ cells per ml. The five liter inoculum is then used to inoculate 160 liters of medium in a 200 liter fermentor to begin a production run.

During the production run, the culture is grown with continuous agitation while infusing $CO_2$ gas at a rate of flow sufficient to maintain a dissolved level of $CO_2$ as determined by a $CO_2$ monitoring probe. A 5–10% level of dissolved $CO_2$ is preferred. The gas is preferably infused as an $N_2/CO_2$ mixture. An 85/15 ratio is preferred, but not critical. The $CO_2$ level may range up to about 50%±10%. The gas is filter-sterilized as it is introduced into the growth chamber. Temperature is preferably controlled at about 37±1° C. The pH is preferably controlled to a substantially constant value ±0.1 within the range of about 6.5 to 7.5 by monitoring with a pH probe/controller and by the addition of KOH as called for by the controller. Fermentation is considered complete when the pH of the culture stops dropping (no more KOH is called for to maintain pH within the set limitation), or when the cell density reaches the high visible density, typically $1–5\times10^9$ cells per ml. At this point the fermentation is terminated by the addition of 100% saturated solution of aqueous trichloroacetic acid to make the fermentation mixture up to a final trichloroacetic acid concentration of about 5%. This may vary.

The addition of trichloroacetic acid to the fermentation broth not only terminates growth by killing the bacteria, but also makes separation of the cells from the broth substantially easier by contributing to flocculation of the cells. The mixture without trichloroacetic acid is very difficult to separate without causing severe disruption of the integrity of both components. Microorganisms and the polysaccharide ie., the hyaluronic acid, do not readily separate by centrifugation or filtration without the trichloroacetic acid addition. Thus, while it is possible to terminate growth of the culture by other means, for instance heat treatment, trichloroacetic acid treatment has the advantage of facilitating subsequent separation of the hyaluronic acid.

The fermentation mixture is pumped from the fermenter through a 0.22 micrometer pore-size Durapore® filtration cassette using the aforementioned Millipore® tangential flow filtration system. This step concentrates the cells from 160 liters to approximately five liters. The filtrate is retained and diafiltered against greater than 10 mega-ohm conductivity reverse osmosis water using a 30,000 nominal molecular weight cut-off Millipore® Pellicon® cassette system until the filtrate, which is continuously discarded, reaches a conductivity of approximately 0.5 mega-ohms. Diafiltering is a powered dialysis technique, such as is disclosed in Catalog Number OM029, March 1981, entitled *Pellicon® Cassette System,* of Millipore Corporation, Bedford, Mass. 01730, as opposed to conventional passive dialysis techniques. The hyaluronic acid is then concentrated by continuing the filtration process without further input of water.

The concentrate is then treated with reagent grade ethanol, preferably in a 3:1 ratio. Other alcohols, acetone, chloroform or other organic solvents as well as certain organic salts such as CETAB, a mixed trimethylammonium bromide, may be used to precipitate the hyaluronic acid or sodium hyaluronate from the aqueous solution. This should be done without any mixing other than occurs in the act of pumping the hyaluronic acid into the solvent. Stirring during alcohol treatment has been found to reduce the process yield of hyaluronic acid. The precipitate at this stage can be stored indefinitely in the dark at 4° C.

As is seen from the procedure described, a unique approach is found in the isolation of the hyaluronic acid from the broth by a two-step process in which a molecular weight separation step is carried out by diafiltration to separate the acid from substantially all of the lower molecular weight components of the broth, and then the acid is separated from any remaining broth constituents by precipitation.

The precipitated hyaluronic acid can be dewatered (removal of the bulk of the water/alcohol solution) by a number of conventional techniques and then resuspended in reverse osmosis water or a 0.15M NaCl solution. The resuspended material is then lyophilized (freeze-dried), spray-dried, vacuum-dried or diafiltered to remove the last traces of alcohol. Further purification is performed by making a 0.05M Borate buffer solution, pH 8.0, with approximately a 10 mg/ml sodium hyaluronate concentration. 0.32% CETAB, is then added to the solution and the mixture stirred at 4° C. overnight to yield a precipitate, sodium hyaluronate. Other precipitating agents may be used, such as cetyl pyridium chloride or related salts. The precipitate is recovered by coarse filtration and resuspended in a 1M NaCl solution made with reverse osmosis water. The resuspended hyaluronic acid is then diafiltered and concentrated as above. The resultant hyaluronic acid can then be filter-sterilized and used or converted to sodium hyaluronate and then be filter-sterilized and used.

Conventional dewatering techniques include pressing, centrifugation, chemical addition and the like. The particular technique selected will depend on the subsequent intended use of the precipitate.

If a medical grade pyrogen-free product is desired, a pyrogen-free filtered growth medium is used and all operations of the process, including the isolation and processing of the hyaluronic acid/sodium hyaluronate are performed under conditions of a class 100 clean room using pyrogen-free containers. If the material is to be used only for chemical grade application, the cleanliness of the room and collection containers is not critical with respect to pyrogens.

The inventive method which emphasizes growing cells under non-aerated conditions prevents the streptococcus from producing its normal complement of end products, primarily the pyrogenic exotoxins for which the microbe is so well known. The described growth conditions also give a much higher yield of hyaluronic acid than has been previously reported. A minimum of 2 grams of hyaluronic acid per liter of culture broth has been obtained using the preferred cell growth and isolation conditions described above. The high yield under the non-aerated conditions is unexpected since one of the proposed functions of hyaluronic acid is thought to be that of providing an oxygen barrier for the cell. Thus, its production would only be expected to be maximized under conditions of exposure to oxygen.

The hyaluronic acid/sodium hyaluronate prepared as described has an average molecular weight of about 55,000± about 20% within a molecular weight range of from about 10,000–2,000,000 as determined by gel filtration or by quasi-elastic light scattering techniques.

These techniques are well known as are the variations in measurement and the results obtained with them due to biological variation. The product also has a protein content of between 0.3% and 0.03% depending on method of analysis. The UV absorption of the 0.1% solution is 0.314 at 260 nm and 0.169 at 280 nm. Viscosity of a 1% solution is approximately 300 centistokes.

A 0.5–1.5 percent solution of the pyrogen-free NaHy produced by the inventive method may be used as an eye drop composition in place of the very dilute solutions of high molecular weight rooster comb derived hyaluronic acid presently used for treatment of keratitis sicca.

Other hyaluronic acid-producing streptococci in the Group A and Group C strains may be used in the invention. Additionally, variations in the growth medium and conditions of growth, as well as variations in the isolation procedures, may be made without departing from the invention which is set forth in the following claims.

What is claimed is:

1. A method of producing hyaluronic acid comprising:
   - fermenting a broth culture of hyaluronic acid-producing streptococcus bacteria in a growth medium under $CO_2$-enriched anaerobic conditions;
   - separating the bacterial cells from the resulting broth; and
   - isolating the hyaluronic acid from the remaining constituents of the broth by separating the acid and those broth consituents of similar or higher molecular weight and then separating the acid from those similar broth constituents by precipitation.

2. A method as in claim 1 wherein the growth medium is made pyrogen-free prior to inoculation by filtration thereof through a 10,000 molecular weight cutoff filter.

3. A method as in claim 1 wherein the bacterial cell separation step includes first adding trichloroacetic acid to the broth.

4. A method as in claim 3 wherein the trichloroacetic acid is added to make the fermentation mixture up to a final concentration of 5–6%.

5. A method as in claim 3 wherein the growth of the bacteria is terminated by said addition of trichloroacetic acid.

6. The method of claim 1 wherein the precipitation is accomplished by adding the hyaluronic acid and the remaining broth constituents to ethanol without substantial mixing.

7. The method of claim 1 wherein isolation is accomplished by separating the broth from the cells, diafiltering the broth and precipitating the acid from solution by adding the solution to an organic solvent without substantial mixing.

8. A method as in claim 1 wherein the fermentation step includes controlling the pH at a substantially constant value $\pm 0.1$ within the range of 6.5–7.5 and the temperature to $37 \pm 1°$ C. throughout fermentation.

9. A method as in claim 8 wherein the pH is controlled by the automatic addition of base with a pH probe/controller.

10. A method as in claim 1 wherein the dissolved $CO_2$ is maintained at a concentration of about 5–10%.

11. A method as in claim 1 wherein the bacteria is grown to a density of greater than $2 \times 10^8$ cells per ml before termination of growth.

12. The method of claim 1 wherein the baceria are *Streptococcus pyogenes* type 18.

13. A method as in claim 1 carried out by filtering the growth medium through a 10,000 molecular weight cutoff filter prior to inoculation with the bacteria culture and performing the remaining steps under conditions of a class 100 clean room using pyrogen-free containers.

14. A method as in claim 13 further comprising: refining the isolated hyaluronic acid by dissolution thereof in a mildly basic buffered solution, precipitation of sodium hyaluronate with a mixed alkyl trimethylammonium bromide, resuspending the precipitate in dilute sodium chloride solution and reprecipitating it as acid by adding it to ethanol without stirring.

15. In the method of producing hyaluronic acid involving production of same by streptococcus bacteria, the improvement comprising isolating the acid from the broth in which the bacteria grows by separating the acid and those broth constituents of similar or higher molecular weight from the liquid and then separating the acid from those similar broth constituents by precipitation, and wherein the precipitation is accomplished by adding the acid and the remaining broth constituents to ethanol without mixing.

16. The method of claim 15 wherein the bacteria are *Streptococcus pyogenes* type 18.

17. The method of claim 15 wherein isolation is accomplished by separating the broth from the bacteria, diafiltering the broth and precipitating the acid from resulting solution by adding the solution to an organic solvent without any substantial mixing.

18. In the method of producing hyaluronic acid involving production of same by streptococcus bacteria, the improvement involving the maintenance of $CO_2$-enriched anerobic conditions in the environment of the bacteria growth medium.

19. A method of producing hyaluronic acid comprising:
   - growing a culture of hyaluronic acid-producing streptococcus bacteria in a growth medium under $CO_2$-enriched anaerobic conditions;
   - forming a liquid suspension of the bacteria and its by-products in the case where the formation of such a suspension is not inherent in the growth method being used;
   - separating the bacterial cells from the suspension; and
   - isolating the hyaluronic acid from the constituents of the remaining liquid by separating the acid and those broth constituents of similar or higher molecular weight and then separating the acid from those similar broth constituents by precipitation.

20. A method as in claim 19 wherein the growth step includes controlling the pH at a substantially constant $\pm 0.1$ value within the range of 6.5–7.5 and the temperature to $37 \pm 1°$ C. throughout fermentation.

21. A method as in claim 19 wherein the pH is controlled by the automatic addition of base with a pH probe/controller.

22. A method of producing hyaluronic acid comprising:
   - growing a culture of acid-producing streptococcus bacteria under $CO_2$-enriched anaerobic conditions on a solid growth medium;
   - forming a liquid suspension of the bacteria and its by-products to separate same from the solid medium,
   - separating the bacterial cells from the suspension; and
   - isolating the hyaluronic acid from the constituents of the remaining liquid by separating the acid and

those liquid constituents of similar or higher molecular weight from the liquid suspension and then separating the acid from those similar constituents.

23. The method of claim 22 wherein the precipitation is accomplished by adding the hyaluronic acid and the remaining constituents to ethanol without substantial mixing.

24. A method as in claim 22 wherein the fermentation step includes controlling the pH at a substantially constant value ±0.1 within the range of 6.5–7.5 and the temperature to 37±1° C. throughout fermentation.

25. A method as in claim 22 wherein the dissolved $CO_2$ is maintained at a concentration of about 5–10%.

26. A method as in claim 22 wherein the bacteria is grown to a density of greater than $2\times10^8$ cells per ml before termination of growth.

27. A method as in claim 22 further comprising: refining the isolated hyaluronic acid by dissolution thereof in a mildly buffered solution, precipitation of sodium haluronate with a mixed alkyl trimethylammonium bromide, resuspending the precipitate in dilute sodium chloride solution and reprecipitating it as acid by adding it to ethanol without stirring.

28. A method of producing hyaluronic acid comprising:

providing hyaluronic acid-producing streptococcus bacteria;

combining the bacteria with a growth medium whereby the bacteria produces by-products including hyaluronic acid;

maintaining $CO_2$-enriched anaerobic conditions in the environment of the growth medium;

forming a liquid suspension of the bacteria and its by-products in the case where the formation of such a suspension is not inherent in the growth method and medium being used;

separating the bacteria from the suspension; and

isolating the hyaluronic acid from the constituents of the remaining liquid by separating the acid and those liquid constituents of similar or higher molecular weight from the liquid suspension and then separating the acid from those similar constituents.

29. The method of claim 28 wherein the bacteria is initially provided in a resting suspension.

30. The method of claim 28 wherein the bacteria is *Streptococcus pyogenes* type 18.

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